A Genome-Wide Knock-Out Screen Identifies Novel Host Cell Entry Factor Requirements for Divergent Adeno-Associated Virus Serotypes

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A Genome-Wide Knock-Out Screen Identifies Novel Host Cell Entry Factor Requirements for Divergent Adeno-Associated Virus Serotypes

A dissertation presented

by

Amanda Mary Dudek

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Virology

Harvard University

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Abstract

Adeno-Associated Virus (AAV) is a non-pathogenic virus that has been harnessed as a vector system for therapeutic gene transfer. Despite decades of research on AAV as a vector, little is known about the molecular determinants required for cellular entry of different AAV capsid serotypes and how this may lead to unique cell and tissue tropism for these viral vectors. This work presents and characterizes a novel AAV entry pathway used by an evolutionarily related subset of divergent AAVs that is independent of the canonical AAV receptor, AAVR, both in vitro and in vivo. This subset of AAV capsids, comprised of rh32.33 and AAV4, are unable to use AAVR for entry or to bind AAVR in vitro. We further characterize the role of AAVR for AAVR dependent serotypes, suggesting a predominantly post-attachment function for this receptor. Mutagenesis of both capsid and AAVR investigate the role human and animal variation may have in the success and translation of AAV-based gene therapies to the clinic. Additionally, a genome-wide CRISPR/Cas9 based entry screen identifies multiple cellular factors required for entry of AAVR dependent and independent serotypes. First, we describe a minimal alternate receptor complex comprised of NEU1 and CTSA that is uniquely required for entry of AAVR independent serotypes and acts at a post-
attachment step in the entry pathway. We also identify and describe a previously uncharacterized protein, required by all AAV serotypes except AAV5. Chimeric capsids suggest both a capsid binding-site as well as an endosomal escape function located within the VP1 unique region of capsid. We therefore present a model in which most AAVs use a canonical entry pathway requiring two conserved entry factors, yet the highly divergent AAV serotypes AAV5, AAV4, and rh32.33 have unique alternate entry factor requirements. Our basic virology studies of AAV aim to both inform currently unanswered questions on the implementation of AAV-based gene therapies in the clinic, as well as allow for better design of AAV-based gene therapies in the future.
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## List of Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAP</td>
<td>Assembly activating protein</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>AAVR</td>
<td>Adeno-associated virus receptor</td>
</tr>
<tr>
<td>ADE</td>
<td>Antibody-dependent enhancement</td>
</tr>
<tr>
<td>ADIN</td>
<td>Antibody-dependent intracellular neutralization</td>
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<tr>
<td>Cas9</td>
<td>CRISPR associated nuclease 9</td>
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<td>CCR5</td>
<td>Cysteine-Cysteine chemokine receptor type 5</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of differentiation 4 glycoprotein</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
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<td>CTSA</td>
<td>Cathepsin A</td>
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<tr>
<td>CXCR4</td>
<td>Cysteine-X-Cysteine chemokine receptor type 4</td>
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<tr>
<td>GPR108</td>
<td>G-protein coupled receptor 108</td>
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<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus type 1</td>
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<tr>
<td>ITR</td>
<td>Inverted terminal repeat</td>
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<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>MANSC</td>
<td>Motif at N terminus with seven cysteines domain</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
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<tr>
<td>NEU1</td>
<td>Neuraminidase 1</td>
</tr>
<tr>
<td>PKD</td>
<td>Polycystic Kidney Disease domain</td>
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<tr>
<td>PPCA</td>
<td>Protective protein/Cathepsin A</td>
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<tr>
<td>RBE</td>
<td>Rep-binding element</td>
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<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>RING</td>
<td>Really interesting new gene domain</td>
</tr>
<tr>
<td>sgRNA</td>
<td>single-guide RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
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<tr>
<td>TM</td>
<td>Transmembrane domain</td>
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<tr>
<td>TRIM-21</td>
<td>Tripartite motif</td>
</tr>
<tr>
<td>TRS</td>
<td>Terminal resolution site</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vesicular stomatitis virus glycoprotein G</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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Dedicated to:

Dr. Kathryn M. Partin, Ph.D.

For being the voice in my head reminding me to “just push through”
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Chapter 1:

Introduction
I: General gene therapy introduction

There are many different human diseases that are known to occur due to genetic mutations. While many diseases such as obesity are thought to have a genetic component, with multiple genes and environmental factors contributing to development of the disease, many are monogenic, meaning that disruption of a single gene is sufficient to cause disease. Often these disorders are recessive and manifest when two copies of a mutated gene are inherited such that the child is missing a functional copy of this gene. This category of diseases includes many prevalent and well-known diseases such as sickle cell anemia, hemophilia, cystic fibrosis, and Huntington’s disease. It is estimated that the worldwide prevalence of monogenic disorders is 1% of all live births, leading to significant morbidity and mortality as well as significant health-care costs to treat these patients.

For many monogenic disorders, the disease can be corrected by re-introducing a functional copy of the gene or the protein product of the gene before major pathology occurs. Patients with hemophilia, for example, are missing a functional copy of a clotting factor needed to form blood clots and therefore are prone to excessive bleeding, which can be prevented by injections of the missing clotting factor. These types of treatments however, can only be developed and successfully implemented when the protein product of the missing gene is functional in systemic circulation (ie: does not have to be taken into the cell in order to function), can be produced exogenously in either a bacterial or mammalian cell culture system, is long-lasting in the patient, and the patient has regular access to these treatments. Additionally, patients develop immunity leading
to toxicity in some cases of protein supplementation, as has been seen for thrombopoietin (1). A more practical and long-lasting solution for many monogenic disorders is instead to provide a functional copy of the missing gene, thereby providing permanent or semi-permanent therapeutic benefit. This is additionally the only feasible option for diseases in which the protein product must be present inside the cell, such as defective structural proteins that cause disease in muscular dystrophy patients (2).

Using either a viral or non-viral delivery method the therapeutic gene can be reintroduced into patients’ cells to provide long-lasting gene expression, and has been

Figure 1.1: Summary of non-viral and viral gene therapy delivery methods. A) Structure of gene delivery vehicle, and B) method of cellular and/or nuclear entry. C) Summary of major advantages and disadvantages associated with each gene therapy delivery method.
proven effective to maintain therapeutic effect for many diseases including hemophilia (3). Non-viral delivery methods often involve either chemical modification of the nucleic acid of interest, such as complex formation into liposomes, or physical force such as electroporation, while viral methods are most commonly modified versions of naturally occurring viruses such as retroviruses, adenoviruses, or adeno-associated viruses [Fig. 1.1.A]. The nucleic acids are then taken into the cell through endocytosis, liposomal fusion, or forced through the membrane for non-viral delivery, or taken into the cell through the viral vector’s natural cellular entry pathway [Fig. 1.1.B]. While non-viral vectors have the advantage of low-cost production and a lack of pre-existing immunity against the treatment, viral vectors are more expensive to produce but are a much more efficient delivery vehicle, as they are inherently great at entering a cell to deposit genetic information [Fig. 1.1.C].

II: Different types of viral vectors

There are several different viruses, predominantly retroviruses, adenoviruses, and adeno-associated viruses that are being developed as gene therapy tools for monogenetic disorders. Each of these viral vectors has their own benefits and drawbacks, and the most suitable vector for a particular indication often depends on the specifics of the disease. Retroviruses encode an RNA genome that is reverse transcribed into a double-stranded DNA genome that stably and irreversibly integrates into the host-cell genome upon cellular entry. Adenoviruses are double-stranded DNA viruses that encode a large genome of roughly 30-40 kb, and are made replication defective in the viral vector context by deletion of one or multiple early genes required
for virus replication. These large genomes however are prone to recombination and can be difficult to produce. Adeno-associated virus is a small single-stranded DNA virus that in its natural form requires co-infection with a helper virus for replication, and encodes only two genes within a genome of only 5kb. Several other viruses have been developed into viral vectors for gene therapy or as anti-cancer therapeutics (oncolytics) such as herpesviruses and measles virus but these vectors are less frequently used for monogenic gene therapies and will not be discussed in detail in this chapter. These viral vectors are generally made by removing genes required for viral replication, replacing them with a therapeutic transgene, and supplying the viral genes required for virus production in trans using up to 3 additional plasmids via co-transfection in cell culture.

As an obligate part of the viral life-cycle retroviruses integrate their genome into the host-cell genome (4) and thus are maintained in the cell as a stable part of the host genome even as the cell divides. This makes retroviruses particularly suitable for treating diseases in which the cells divide many times throughout the patient’s life. While genomic integration can be advantageous when targeting cells that have a high turn-over rates such as immune cells, it may lead to insertional mutagenesis, causing malignant transformation of the transduced cells. In fact, early gene therapy trials for X-linked severe combined immunodeficiency (SCID) using retroviral gene therapy vectors hit major roadblocks when several treated children developed leukemia due to an unfavorable viral integration event leading to inactivation of a proto-oncogene (5-7). Conversely, Adeno-associated viral vectors (AAV) do not have obligate integration as part of their transduction pathway (8), have a more desirable safety profile. In slowly or
non-dividing cells such as in the retina, adeno-associated viruses have been shown to be long-lived in animal models, with sustained gene expression for months or years.

Adenovirus and adeno-associated virus are both DNA viruses, but vary greatly in genome size and complexity. While human adenoviruses contain 10 transcription units, each giving rise to multiple proteins in a total genome size of ~35kb, adeno-associated virus only encodes two genes in its 5kb genome. While adenovirus has the advantage of a large packaging capacity, adenovirus highly activates innate immune responses, which can be dangerous when given in the large doses required for viral-based gene therapy. Patients may often have neutralizing antibodies against the most common AAV serotypes, but it uniquely has little to no activation of innate immunity compared to many other viruses, making it a highly attractive and safe gene therapy vector. Viral variation of both adenovirus and adeno-associated virus gives rise to different serotypes, which each have distinct tissue and cell targeting properties as well as different immunogenic properties. People have attempted to work around the natural human immunity against these vectors by using viral vectors that are less seroprevalent in the population, and by attempting to modify capsid to evade neutralizing antibodies.

Adeno-associated virus is an attractive viral vector due to its safety profile and has demonstrated efficacy in the clinic, amassing a growing list of successful clinical trials and leading to the first FDA approved viral gene therapy in the United States. While AAV has a small packaging capacity, there has been promising work to expand the utility of these vectors to treat diseases caused by mutations in larger genes as well.
Dual-AAV vectors in which each half of the transgene of interest is delivered in two separate viral vectors and combines inside the cell have shown promise in cell culture and to an extent in animal models of disease. Additionally, attempts to generate smaller functional versions of a therapeutic gene have led to successful treatments such as mini-dystrophin and micro-dystrophin therapy for muscular dystrophy. AAV has also been used in combination with CRISPR/Cas9 ex-vivo for gene editing of hematopoetic stem cells for various blood and immune disorders, a method which both negates the problem of pre-existing immunity as well as small packaging capacity of the virus. Development of these creative new methods to apply AAV-based gene therapies are allowing us to broaden the types of diseases that can successfully be treated with gene therapy.

III: Translational and clinical development of AAV gene therapies

As a natural human virus with no known pathogenesis, AAV is an ideal candidate for development as a gene therapy delivery vehicle, and many studies have demonstrated clinical success with this vector. Development of AAV gene therapy vectors has recently led to the first FDA approved gene therapy treatment, Luxturna (9), for a type of genetic retinal degeneration caused by mutations in the RPE65 gene. The success of this and other AAV-based therapeutics is due to the favorable safety profiles of AAV compared to other viral gene therapy vectors. AAV has been shown to provide therapeutic levels of gene transfer for a multitude of indications for which the therapeutic gene of interest is small enough to be contained within the small capacity of the vector. Current AAV-based gene therapy vectors lack Rep proteins and the rep binding site, so the AAV
transgene primarily exists as an episome (10-12), and the potential for viral integration leading to toxicity is very low. Although there was a recent study suggesting that AAV-like sequences can be found in human liver tumors (13), the development of tumors after AAV injection has not been observed in animal models or in countless clinical trials in humans and thus suggests this is not a major concern for patients. Based on these and other data, some claim that AAV infection may actually lead to an anti-cancer effect (14). Adenoviral gene therapy vectors are also in development, but the robust immune response activated by adenovirus causes concern. Immune activation led to the death of a patient in a clinical trial being treated for ornithine transcarbamylase deficiency after the infusion of a large systemic dose of adenoviral vector caused a cytokine storm leading to multiple organ failure (15). Importantly, AAV is a unique virus in its low immunogenicity, and lack of activation of a robust innate immune response. In the clinic, patient immune responses to AAV-based vectors have been mild and are observed by concomitant elevation of liver transaminase levels and drop in transgene expression (16), suggestive of immune destruction of AAV-transduced hepatocytes in the absence of immunosuppression. These responses however can be well controlled with a concurrent course of steroid treatment (17), often now given prophylactically as oral prednisolone when administering AAV systemically such as in a recent highly successful clinical trial for Type-I spinal muscular atrophy (18). Due to its favorable safety profile, AAV is a highly attractive candidate for developing viral vectors, yet there are still holes in our understanding of the transduction biology relevant to vector targeting and other host-vector interactions that determine its safety and efficacy profile.
IV: Adeno-associated virus biology

Adeno-associated virus is a small, roughly 20nm, non-enveloped linear single-stranded DNA virus. AAV is part of the dependovirus genera of parvoviruses, and was originally identified as a contaminant in an adenovirus preparation (19, 20). It is not known the exact transmission route of the wild type virus, but it is thought to be through either the respiratory or gastrointestinal routes. AAVs have been isolated from multiple different human tissues including hematopoetic cells (21, 22), male (23) and female (24, 25) reproductive tissues, and frequently in muscle biopsies (26).

For efficient viral replication these dependoviruses require co-infection of the same cell with a helper virus, most commonly adenovirus (19, 20, 27), but herpesvirus (28-30), vaccinia virus (31, 32), and human papillomavirus (33-35) have also been shown to have helper functions. The negative sense viral genome is about 5 kilobases, and contains two genes, flanked by palindromic Inverted Terminal Repeat sequences (ITRs) that allow packaging of the genome into the viral capsid (36) as well as serving as primers for viral DNA synthesis (37) [Fig. 1.2.A]. The Rep gene encodes multiple proteins that serve the genome replication and packaging functions not provided by the host cell, and the Cap gene encodes the structural proteins required for assembly. The AAV life-cycle allows persistent latent infection either as episomal DNA (38, 39), large molecular weight concatemers (40, 41), or as a site-specific integrant into the host cell DNA mediated by the Rep protein (8) which can be re-activated by co-infection with a helper virus (42).
The 3’ end of the AAV genome contains the rep gene, which encodes the viral non-structural proteins required for genome replication and packaging that are not provided by the cell or helper virus. These four non-structural proteins are named for their apparent molecular weight, Rep78, Rep68, Rep52, and Rep40 [Fig. 1.2.B]. Alternative splice variants generate the two larger rep proteins, Rep78 and Rep68, expressed from the p5 promoter in the 5’ ITR. The functional distinction between these two proteins is unclear, as they have been shown to have the same molecular functions in genomic DNA hairpin binding (43-45), nicking of genomic replication intermediates (46, 47),
helicase activity (48, 49) and either repression of the p5 (50) or transactivation of the p40 promoter (51). Functional differences between the two smaller rep proteins, Rep52 and Rep40, are also unclear as they can both bind ATP and possess helicase activity (52, 53). It is thought that the smaller rep proteins facilitate packaging of the genome into pre-formed capsids through the pore at the 5-fold axis of symmetry (53), and remain covalently bound to the 5' end of the DNA genome (54) as the analogous protein, NS1, does for other parvoviruses (55, 56). Rep 52 was also shown to repress the p5 promoter, but to a lesser extent than Rep78 or Rep68 (50).

The 5’ end of the AAV genome encodes cap, a gene encoding two open reading frames from which all structural and assembly proteins are expressed. Three different structural proteins, VP1, VP2, and VP3, are expressed from the same open reading frame as alternate splice variants and through leaky translation initiation [Fig. 1.2.C]. These proteins share their c-terminus and are expressed at a 1:1:10 ratio in the assembled capsid, such that the capsid 60-mer is primarily composed of VP3. While VP3 is sufficient to assemble virus-like particles, VP1 and VP2 are required for productive infection due to the viral phospholipase (vPLA) domain (57) and basic repeats (+) (58) which serve as nuclear localization signals. Recently another structural protein, Assembly Activating Protein (AAP), was identified from a small alternate open reading frame (59), and is required for assembly of most (but not all) AAV serotypes (60-62). This protein has no homology with other known proteins and appears to be disordered, although some functional domains have been mapped which dictate capsid’s requirement on AAP (62-64). As the outer protein coat, the cap gene determines
antigenicity, or serotype, of the virus. Throughout the past decades there have been many identified and described capsid serotypes with unique biology and tissue targeting aspects (for further discussion see section VII). However, due to antibody cross-reactivity and high level of engineered variation within this gene for viral vector studies, the term “capsid variants” may rather be better suited than the more commonly used term “serotype”.

Rep and Cap are flanked by inverted terminal repeat (ITR) sequences that facilitate replication of the wild type genome, and packaging of wild type or vector genome [Fig. 1.2.A]. The ITR is 145 nucleotides long and is highly palindromic, causing 125 bases of complementarity to form a T-shaped hairpin structure at each end of the viral genome (65). The 3' terminus serves as a primer from which the cellular DNA polymerase generates double-stranded DNA intermediates (66, 67) that are resolved by Rep68/78 binding at the rep-binding site (RBS) and cleavage of the terminal resolution site (TRS). In the absence of nicking, the genome can form head-to-head or tail-to-tail concatemers (37). It is thought that in an AAV vector context concatemeric forms of the genome facilitate transgene expression, as high molecular weight DNA can be observed in injected animals with long-term transgene expression (40, 68), yet this remains to be definitively proven.
V: Adeno-associated virus entry and trafficking pathways

AAV undergoes attachment at the cell surface via serotype-specific glycans (69-74) [Fig. 1.3.A]. AAV2 for example binds to heparin sulfate proteoglycan (71, 75, 76), and AAV4 binds to o-linked sialic acid (73). Although the presence of these glycans clearly influence the overall transduction level of their cognate serotype, it is not an absolute requirement for infection, as an AAV2 capsid whose surface residues are
mutated to no longer bind heparin sulfate is still infectious, although to a decreased extent both in vitro and in vivo (77). Interestingly, live-cell imaging of AAV2 infection of HeLa cells suggests that the virion comes into contact with the cell an average of 4.4 times before undergoing endocytosis (78). There have been many reported protein receptors or co-receptors in the literature thus far (79-86). These putative receptors however, often only show an increase in transduction upon overexpression, but are not a major requirement for entry and thus should be more readily classified as attachment factors. For a detailed breakdown of putative glycan and protein attachment factors and entry receptors, see Table 1.1.

Table 1.1: Summary of proposed glycan and protein attachment factors (previously termed “receptors”) and entry receptors for a variety of AAV serotypes

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Glycan attachment factor</th>
<th>Protein attachment factor</th>
<th>Entry receptor</th>
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<tbody>
<tr>
<td>AAV1</td>
<td>N-linked sialic acid</td>
<td>Unknown</td>
<td>AAVR</td>
</tr>
<tr>
<td>AAV2</td>
<td>Heparin sulfate proteoglycan</td>
<td>FGFR, HGFR, integrins, CD9, LamR,</td>
<td>AAVR</td>
</tr>
<tr>
<td>AAV3</td>
<td>Heparin sulfate proteoglycan</td>
<td>HGFR, LamR</td>
<td>AAVR</td>
</tr>
<tr>
<td>AAV4</td>
<td>O-linked sialic acid</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>AAV5</td>
<td>N-linked sialic acid</td>
<td>PDGF</td>
<td>AAVR</td>
</tr>
<tr>
<td>AAV6</td>
<td>Heparin sulfate proteoglycan, N-linked sialic acid</td>
<td>EGFR</td>
<td>AAVR</td>
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<td>AAV7</td>
<td>Unknown</td>
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After attachment, AAV2 undergoes rapid endocytosis, within 100ms (78). There are still debates over whether endocytosis [Fig. 1.3.B] is clathrin dependent or independent. Clathrin mediated entry was disrupted by preventing clathrin lattice formation using chlorpromazine (CPZ), which showed a 2-fold decrease in AAV2 transduction in HeLa cells (87). Overexpression of a dominant negative dynamin I mutant, K44A, was also shown to reduce AAV2 transduction 5-fold in HeLa cells (88). Conversely, a thorough study using different entry inhibitors concluded that rather than clathrin mediated endocytosis, AAV2 used the CLIC/GEEC (clathrin-independent carriers/GPI-anchored-protein-enriched endosomal compartment) pathway which is clathrin, dynamin, and caveolin independent (89). Macropinocytosis has also been reported to play a role in AAV2 entry, as inhibition of this pathway using a dominant negative form of Rac1, N17Rac1, also slightly decreased AAV transduction (90). Observed endocytosis differences in various studies are thought to be due to different particle-to-cell ratios used in these studies, as CPZ treatment had no effect on the inefficient AAV2HSPG-mutant at $2 \times 10^3$ particles per cell, but inhibited entry at $5 \times 10^3$ particles per cell (87). This aspect of the viral entry pathway however, remains in question.

Trafficking to the nucleus is thought to occur in a microtubule-dependent fashion [Fig. 1.3.C], yet disruption with nocodazole decreases AAV2 transduction only 2-fold (91). However, live-cell imaging studies of fluorescently labeled virus demonstrate AAV2 colocalization with microtubules (91), and individual AAV2 virions have been observed within trafficking endosomes (78, 91). While in the endosome, cellular cathepsins [Fig. 1.3.D] may cleave some AAV serotypes in a sequence-specific manner (92). This
cleavage is thought to facilitate genome uncoating, but the lack of cellular cleavage of most capsid serotypes suggests that this is not an obligate part of the entry pathway.

The phospholipase domain within the VP1 unique region is thought to facilitate endosomal escape [Fig. 1.3.E], but it is unclear at what stage of the endosomal pathway this occurs. Observation of AAV trafficking within membranous vesicles, similar perinuclear accumulation of VP1 phospholipase mutant and normal AAV2 particles (57), and required trafficking to the trans-golgi network (93) suggest that endosomal escape happens late in the endocytic pathway. Processing of the viral capsid in the endosome either through acidification leading to VP1/2 extrusion and/or cleavage by cellular proteases (92) is thought to be required for productive infection. This hypothesis is elegantly supported by experiments demonstrating that microinjection of AAV2 into the nucleus causes a greater than 10-fold decrease in transduction, and microinjection into the cytoplasm causes a greater than 100-fold decrease in transduction as measured by %Rep positive cells (94). Some reports suggest that importin-β facilitates nuclear import [Fig. 1.3.F], but there is only a 2-fold decrease in infectivity upon importin-β knock-down (95). Uncoating of the genome is thought to occur in the nucleus [Fig. 1.3.G], as intact AAV2 particles can be visualized in the nucleus and nucleolus using an antibody which only recognizes assembled capsids (96), yet it has not been proven whether these visualized particles undergo productive infection or not. In the absence of efficient nuclear import, the vector will be degraded by the proteasome [Fig. 1.3.H], and protease inhibitors have demonstrated varying levels of increase in transduction depending on the capsid serotype in question (97-102).
It is extremely important to highlight that almost all of these studies have been done on AAV2, and modest effects have been used to interpret major mechanistic conclusions, as well as extrapolate these findings to generalize the entry mechanism used by all AAVs.

There is little known about the differences in the cell biology behind the entry process for different AAV serotypes that lead to specific targeting properties observed \textit{in vivo}. Most mechanistic studies have thus far been done on AAV2, and these findings are then extended to the majority of other serotypes when they in fact behave very differently from AAV2 both \textit{in vitro} and \textit{in vivo}. Only recently, in 2016, was a bona-fide AAV entry receptor identified and demonstrated to be required for entry of all tested AAV serotypes using a haploid genetic screen (93). The previously uncharacterized protein, KIAA0319L, was re-named AAVR for its function as an AAV receptor, and was shown to be absolutely required for AAV entry both \textit{in vitro} in multiple cell lines, and \textit{in vivo} in an AAVR KO mouse. SPR experiments on purified AAVR ectodomain and AAV vector demonstrated direct binding of capsid to AAVR, and subsequent viral overlay studies mapped this interaction to PKD2 domain of AAVR, with the exception of AAV5, which binds to PKD1 (103). Antibody-blocking experiments preventing AAV entry suggest a potential role in AAV attachment, and swapping of the c-terminal trafficking domain with other endosomal proteins also suggests a major role in trafficking through the endosomal system to the correct sub-cellular compartment for entry. While these
studies were novel in their identification of a highly conserved AAV entry receptor, this study was ironically also done using AAV2.

VI: The adeno-associated viral vector system

Although co-infection with a helper virus increases both early and late stages of viral replication, it is not a strict requirement for viral replication (104) and so this virus can be easily developed into a viral vector system in the absence of an actively replicating helper virus. In the vector context, the endogenous rep and cap genes are removed

Figure 1.4: Diagram of AAV vector system. (A) Endogenous Rep and Cap genes are replaced by a therapeutic transgene or reporter transgene [eg: Luciferase] and an application-specific promoter [eg: CMV]. Rep and Cap are provided in Trans from pAAVector and helper functions are supplied by pAdF6 via co-transfection in HEK293 cells (B). (C) Cells are lysed to release vector via freeze-thaw, and genome-containing vector is purified by tangential-flow filtration [TFF] and iodizanol gradient ultracentrifugation. (D) Packaged transgene is delivered to and expressed in the target cell after AAV entry.
from the viral genome, and replaced by a transgene of interest, (36) with initial proof-of-concept studies having been done using β-galactosidase (105). This ITR-flanked transgene is then expressed in trans with a construct encoding rep and cap, plus a construct that provides necessary helper function, generally in the form of several adenoviral proteins [Fig. 1.4.A]. This system can be used to produce AAV from mammalian (most often HEK293) cells [Fig. 1.4.B], or can be adapted to a baculoviral system (106). When designing viral vectors, the transgene is often driven by a tissue specific promoter to prevent pathology due to expression in incorrect cell types [Fig. 1.4.A]. To produce the viral vector, the therapeutic transgene flanked by the AAV ITRs can be co-expressed in cells in trans with rep, cap, and several adenovirus helper virus proteins in order to produce a replication defective adeno-associated virus vector [Fig. 1.4.A]. After purification [Fig. 1.4.C], this vector can be delivered systemically or via a targeted delivery method when appropriate. These vectors can be delivered [Fig. 1.4.D] as a vector containing a single-stranded viral genome, for a total packaging capacity of roughly 4.5 kB. Larger constructs demonstrate decreased viral production and packaging of truncated genomes due to the structural constraints of the capsid (107, 108). Transgene expression can also be increased in some cases using self-complimentary AAV vectors, in which the viral vector contains a reverse-complement sequence of the transgene (109). This sequence anneals on itself to generate a double-stranded template for more rapid gene expression, but the packaging capacity is decreased to about 3.3 kB. The AAV system is easily adaptable to different genetic indications in which gene-replacement therapy is sufficient, as the specific AAV capsid serotype, transgene, promoter and other regulatory elements are easily interchanged.
VII: The importance of capsid serotype in AAV gene therapy development

AAV-based gene therapies are currently under development using a variety of different AAV capsids isolated from various sources, primarily humans and different species of non-human primates (110, 111). The most commonly used and well-studied AAV serotypes are AAV2, AAV8, and AAV9, and targeted therapies are developed based on the specific tissue-targeting properties of these capsids. AAV2 is often used for treatment of retinal diseases and is the serotype in which Luxturna was developed and approved (9). AAV8 shows high levels of liver targeting, and is under development for both liver diseases or diseases in which a secreted factor is produced such as hemophilia (3, 17). AAV9 efficiently targets the central nervous system (112, 113), and has shown striking results including drastically increased survival rates in a recent trial of gene-replacement therapy for Type-I spinal muscular atrophy (18, 114), a disease that is fatal in young children. Although these different capsids have been well studied as gene therapy tools, it is poorly understood what determines targeting of these different capsids to specific cells and tissues on a cellular level.

Aside from these commonly used serotypes, there are hundreds of different capsids that have been identified and are under development as gene transfer vectors. These capsids have been isolated in biomining efforts in human and non-human primate tissues (110), or novel capsids have been generated from known capsid sequences using different methods including error-prone PCR (115), directed evolution (115), capsid shuffling (116), peptide insertion (117), and ancestral sequence reconstruction
Multiple unnatural variants are used throughout the subsequent chapters, the details of which are summarized in Table 1.2. While these methods have developed many novel capsids that facilitate further development of gene therapies with desirable properties such as targeting of a specific cell type (120) or evasion of neutralizing antibodies (121), it is often poorly understood what leads these variants to have these properties. Thus far, targeting studies have primarily been done by injecting many different capsid variants containing a reporter gene such as GFP or luciferase through

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different routes of administration to determine cell and tissue tropism of the capsid (122).

**VIII: Significance**

Our studies aim to probe the effect that specific cellular factors have on AAV entry, and to systematically uncover differences and similarities in entry pathway usage between different serotypes to facilitate better gene therapy implementation in the following ways. We aim to better understand the effect that serum proteins and antibodies have on the intracellular entry pathway leading to vector efficacy in vivo. We have also studied the effect that human and animal genetic variation in required entry factors may have on implementation of gene therapies in people and development of the most relevant gene therapy animal models. We additionally provide evidence for unique entry pathways employed by different serotypes that can potentially be used to understand tropism of the different AAV vectors under development, as well as allow rational design of viral vectors with specific desired features in the future.
Chapter 2:

Identification and characterization of an alternate, AAVR-independent, route of adeno-associated virus entry
I: Abstract:
Determinants and mechanisms of cell attachment and entry steer the adeno-associated virus (AAV) in its utility as a gene therapy vector. Thus far a systematic assessment of how diverse AAV serotypes engage their proteinaceous receptor AAVR (KIAA0319L) to establish transduction has been lacking, despite potential implications for cell and tissue tropism. Here, a large set of human and simian AAVs as well as in silico reconstructed ancestral AAV capsids were interrogated for AAVR usage. We identified a distinct AAV capsid lineage comprised of AAV4 and AAVrh32.33 that can bind and transduce cells in the absence of AAVR, independent of multiplicity of infection. Viral overlay assays and rescue experiments in non-permissive cells demonstrate that these AAVs are unable to bind to or use the AAVR protein for entry. Further evidence for a distinct entry pathway was observed in vivo, as AAVR knock out mice were equally permissive to transduction by AAVrh32.33 compared to wild type mice upon systemic injection. We interestingly observe that some AAV capsids undergo a low level of transduction in the absence of AAVR, both in vitro and in vivo, suggesting that some capsids may have a multi-modal entry pathway. In aggregate, our results demonstrate that AAVR usage is conserved amongst all primate AAVs except for those in the AAV4 lineage, and a non-AAVR pathway may be available to other serotypes. This work furthers our understanding of entry of AAV, a vector system of broad utility in gene therapy.

II: Introduction:
Adeno-associated virus (AAV) is a small, non-enveloped single stranded DNA virus that is not associated with any known human pathogenesis. As such, AAV is an attractive
vehicle for therapeutic gene transfer. AAV gene therapy has been used safely and effectively for multiple indications, such as hemophilia (3, 123) in clinical trials. An AAV drug developed for treatment of Lipoprotein Lipase deficiency (124-126) was the first licensed AAV-based therapy (Glybera) in Europe, however was taken off the market. Other AAV gene therapies such as for Spinal Muscular Atrophy (114, 127, 128) and Leber Congenital Amaurosisis (129-132) are more promising and also on track to possible drug approval. Although great strides have been made in the translation of AAV gene therapy into the clinic, there are still large voids in our understanding of the host factors determining AAV entry and tropism.

AAV is known to attach to specific glycans at the plasma membrane in a serotype-dependent manner (69-74). Several reports have identified putative protein receptors, however the role of these were more incremental and with little demonstration of their relevance in vivo (79-86). After attachment the vector undergoes endocytosis and microtubule-dependent trafficking (91) to the nucleus. It remains unclear whether endocytosis is clathrin dependent (87, 88) or independent (89). After internalization, the AAV particle exits the endosome using the phospholipase domain located in the VP1 portion of the capsid (57). AAV nuclear import is thought to be mediated by importin-β, but siRNA knock-down only causes a moderate decrease in transduction efficiency (95). Of note, most of our mechanistic understanding comes from study of only a few serotypes, most notably AAV2, so it is unclear what serotype specific entry pathway differences dictate the unique biodistribution patterns observed in vivo.
Recently a multi-serotype AAV receptor (AAVR) was identified using an unbiased genome-scale genetic screen (93). AAVR directly interacts with AAV particles via Ig-like PKD domains present in the ectodomain of AAVR (93, 103). It is required for efficient transduction of multiple AAV serotypes (AAV1, 2, 3B, 5, 6, 8, 9) in all cell lines that were tested. Furthermore, AAVR is essential for in vivo transduction as demonstrated with AAV9 using an AAVR knock out mouse. Because AAVR is a rapidly endocytosed receptor that traffics to the Golgi, AAVR could play a role in cell attachment, trafficking of AAV particles to the Golgi network or escape of the AAV out of the Golgi network. However, the exact role of AAVR in these processes is still unknown as is its relationship with the glycan receptors that are important for cell surface attachment.

In the present study, we examine from an evolutionary perspective how different AAV capsid lineages require AAVR and further define the role of AAVR in facilitating cell attachment. We found that the majority of the serotypes are strongly dependent on AAVR for transduction and that AAVR likely acts post-attachment. Our analysis includes in silico reconstructed ancestral AAV capsids, which differ significantly from the extant strains suggesting that AAVR-dependence has arisen early. Strikingly, we identified a single AAV4-related evolutionary lineage that has evolved independence from AAVR, and several capsids that are able to enter cells through an alternate pathway in the absence of AAVR. Representative members of this AAV4 lineage efficiently transduce AAVR knockout cell lines derived from different cell types as well as mice that lack AAVR. Our work highlights the conserved nature of AAVR amongst most primate AAVs
yet identifies several scenarios in which transduction is achieved in the absence of AAVR.

III: Results

Identification of AAVR independent serotypes.

Since the initial discovery of AAV, there have been over a hundred different naturally occurring isolates and countless other capsid variants identified either through rational design, error-prone PCR, or capsid shuffling, each with their own unique cell and tissue tropism profile (110, 111). We examined AAVR usage in Huh7 cells, a human hepatocyte-derived cellular carcinoma cell line, with (WT) or without (KO) AAVR expression in a transduction assay using replication-defective AAV expressing a luciferase transgene, and assessed a total of 9 natural isolates, 2 directed evolution variants, and 7 putative ancestral variants, most of which had not been previously tested [Fig 2.1A]. Most of the AAVs tested demonstrated a strong requirement for AAVR, illustrated by a greater than ten-fold decrease in transduction of the KO vs. WT cells [Fig. 2.1.B]. Interestingly, however, a chimera of two rhesus isolates, AAVrh32.33 (133, 134) and an African green monkey isolate AAV4 (135, 136) were uniquely able to transduce AAVR KO cells as efficiently as WT cells. These data illustrate that the majority of AAV serotypes, yet not all, primarily use AAVR for entry.
AAV4 and AAVrh32.33 represent a distinct evolutionary lineage that is independent of AAVR.

Since AAV4 and AAVrh32.33 are highly divergent from many other currently circulating...
serotypes, we decided to interrogate a panel of capsids predicted to be evolutionary intermediates (118) in an attempt to identify where AAVR independence may have arisen [Fig 2.1.A]. Although many of the putative ancestral capsids are highly divergent from the extant serotypes, they also demonstrated a high level of AAVR usage in the same assay, with some serotypes such as AAVAnc80 having a greater than 1,000 fold decrease in transgene expression [Fig. 2.1.B]. These data demonstrate that AAV4 and AAVrh32.33 represent a distinct evolutionary lineage of AAV capsid that can undergo AAVR independent entry [Fig. 2.1.A].

**Peptide engraftment does not alter AAV receptor usage**

Regions of capsid in hypervariable region IV, near the heparan sulfate binding motif of AAV2 have been shown to tolerate a small peptide insertion, which often leads to a unique cell or tissue type specificity. This begs the question whether peptide insertion allows usage of a secondary entry receptor. We examined two peptide insertion mutant capsids, AAV7M8 which has demonstrated increased outer retinal transduction via intravitreal injection (137), and AAV8BPV2 which transduces retinal bipolar cells (120). Flow cytometry of Huh7 WT and AAVR KO cells transduced with CMV.eGFP transgene were tested and both peptide insertion mutants required AAVR for efficient entry, demonstrating that AAVR is required to complete the viral entry process in both parental and peptide insertion capsids [Fig. 2.1.D]. Although transduction was highly decreased in AAVR KO cells, we were still able to observe transduction of some cells by both AAV7M8 and its parental capsid, AAV2 [Fig. 2.1.E]. Similar levels of transduction in
these two serotypes (about 3% [Fig. 2.1.D]) suggest that peptide insertion may influence another aspect of cellular targeting or entry rather than engagement of its protein receptor.

**AAVR dependence is transgene and helper virus independent.**

Since AAVR has been shown to directly bind intact capsid, it is not surprising that AAV2 capsids expressing different transgenes, either Luciferase [Fig. 2.1.B] or eGFP [Fig. 2.1.D] were both defective for transduction in AAVR KO cells. This further demonstrates that AAVR-mediated entry is an inherent property of the capsid. Since the presence of a helper virus increases AAV transduction, we wanted to know whether co-infection with helper virus would alter AAVR usage. Huh7 WT or Huh7 AAVR KO cells were pre-incubated with 200 pfu/cell of WT hAd5 for 2h prior to AAV transduction with CMV.Luciferase or CMV.eGFP.T2A luciferase transgene, and transduction efficiency was assessed by luciferase assay. While overall transduction increased in all conditions [Fig. 2.1.C], the AAVR usage trends remained the same, with all serotypes aside from AAV4 and AAVrh32.33 maintaining a high level of dependence on AAVR. However, highly reduced yet detectable transduction was seen for AAV3 and AAV6.2 in the absence of adenovirus co-infection, and for AAV1, 2, 3, 6.2, 8, 7m8 and some other AAVs at lower levels [Fig 2.1.B, C, E] indicating the possibility of low level of AAVR-independent entry for those serotypes. These data suggest that AAVR usage is inherent in the capsid and that it is independent of transgene or helper virus.
AAVR independent entry is a pathway that exists in multiple cell lines. Previous studies have demonstrated that AAVR is required for entry of many AAV serotypes, an observation that was confirmed in multiple cell lines (93). Using a luciferase assay to assess AAV entry and subsequent transgene expression, we confirmed that AAV2 entry is abolished in A549 and HeLa [Fig. 2.2.A]. A highly reduced

![Graph A](image)

**Figure 2.2:** **AAVR independent entry is serotype specific and exists in a variety of cell types.** A) *In vitro* transduction of 10,000 VG/cell AAV2, 10,000 VG/cell AAV4, and 100,000VG/cell AAVrh32.33 with CMV.Luciferase.SVPA transgene in A549, HeLa, and Huh7 WT or AAVR KO cells. Data presented are mean +/- SEM of 3 independent experiments. B) Dose response of cells transduced with AAVrh32.33, AAV6.2, or AAVAnc80 CMV.Luciferase.SVPA over a 5-log range of viral genomes per cell in Huh7 WT or Huh7 AAVR KO cells
yet low level of luciferase expression in Huh7 AAVR KO cells was retained, demonstrating the strong dependency of AAVR for transduction of AAV2 however a possible alternate entry pathway that is available in some cells for some serotypes. In all tested cell lines there were comparable levels of transduction by AAV4 and AAVrh32.33 in the WT and KO cells [Fig. 2.2.A]. Entry of AAV4 and AAVrh32.33 into AAVR KO A549, HeLa, and Huh7 cells derived from different tissues (human lung carcinoma, human cervical cancer, and human hepatocellular carcinoma, respectively) suggests that these diverse cell types express an alternate protein receptor that these vectors are able to use for viral entry.

**AAVR independence is an alternate AAV entry pathway.**

Previous studies of AAV2 entry have produced conflicting reports on receptor usage and endosomal pathway requirements; differences in which are thought to be due to different MOIs used for these studies (87-89). Due to this and the low level of transduction observed by some serotypes in AAVR KO cell lines [Fig. 2.1.A], we wanted to test whether this observation was due to capsid specific differences in receptor usage, or whether it was non-specific uptake of highly potent vectors. To test this, we assessed transduction of several serotypes over a 5 log range in viral genomes per cell. Interestingly, we observed three distinct scenarios. First, our previously identified AAVR independent serotype, AAVrh32.33 was able to undergo similar levels of transduction in AAVR KO and WT cells at all viral doses tested [Fig. 2.2.B]. Second, we observed an intermediate phenotype in which some vectors, such as AAV6.2, could undergo significant levels of transduction in AAVR KO cells at high viral doses. Third, many
vectors such as AAVAnc80 were unable to transduce AAVR KO cells at any viral dose tested, despite very high levels of transduction in WT cells. These different phenotypes suggest that there is an alternate entry receptor used by AAVrh32.33, and that some serotypes are able to use this alternate pathway, although for these serotypes it is much less efficient than in AAVR-expressing cells. This data is in line with previously presented data suggesting that some AAV serotypes such as AAV2.5 may be able to undergo entry in the absence of AAVR on polarized airway epithelial cells (138).

**AAVR expression is not required for cellular attachment**

To distinguish between a role of AAVR in AAV attachment and a role in post-attachment steps such as endocytosis, trafficking or endosomal escape (93), we employed a cellular binding assay in AAVR overexpressing cells and AAVR knockout cells. To further assess the role of AAVR in the absence of potentially dominant glycan interactions, we pretreated the cells with neuraminidase to cleave the terminal sialic acid from cellular glycans, or virions were pre-treated with soluble heparin, which competes for heparan sulfate proteoglycan binding [Fig. 2.3.A]. As expected for the AAVR-independent serotypes AAV4 and AAVrh32.33, no differences were observed between AAVR overexpressing or knockout cells in binding [Fig. 2.3.B] or transduction [Fig. 2.3.C]. In agreement with previous studies and validating our binding assay, AAV4 binding was reduced by neuraminidase treatment [Fig. 2.3.B] (73). Interactions with heparan sulfate or sialic acid do not play a major role for AAVrh32.33 binding [Fig. 2.3.B] (139), or are redundant. It is of note that AAV4 and AAVrh32.33, which interact
differently with glycans are both independent of AAVR. Importantly, we did not observe

Figure 2.3: **AAVR is not required for attachment or entry of AAVR independent serotypes.** A) Diagram of *in vitro* cell-binding assay. Paired binding (B) and transduction (C) assays for $10^4$ VG/cell AAV4, AAVrh32.33, and AAV2 in HEK293 AAVR overexpression (rescue) or HEK293 AAVR KO cells. Data presented are one representative experiment of three independent trials and standard deviation of technical replicates.
differences in binding of AAV2, which uses AAVR, in AAVR overexpressing versus knockout cells even when the transduction was performed in the presence of soluble heparin, which decreased AAV2 binding and transduction by ten-fold. Transduction of AAV2 assessed in parallel showed a greater than 100-fold decrease in transduction of the KO cells [Fig. 2.3.C], demonstrating that AAVR expression is not a requirement for AAV2 attachment. Although we observe similar levels of AAV2 binding in both WT and KO cells, AAV2 may still first interact with AAVR at the cell surface, however this interaction would be indistinguishable in this assay.

**AAV4 is unable to bind purified AAVR domains or AAVR expressed in cell lysates.**

Although there is no observable role for AAVR in cellular attachment of AAV4 and AAVrh32.33, we wanted to determine whether these vectors are able to bind to AAVR even if unable to functionally engage it for cellular entry. We used a previously published viral overlay assay (103, 140) to interrogate binding of AAV4 to purified AAVR or AAVR expressed in cell lysates. AAV4 viral overlay on purified AAVR domains illustrates that under the same conditions demonstrating AAV2 binding to AAVR, no detectable binding is observed for AAV4 to AAVR’s PKD1, PKD2, PKD3, or PKD1-5 [Fig. 2.4.A]. Conversely, AAV2 is able to bind purified PKD2 as well as PKD1-5 [Fig. 2.4.B], as previously published (103). Anti-His [Fig. 2.4.C] and anti-AAVR [Fig. 2.4.D] western blots demonstrate that these constructs are efficiently detected on the PVDF membrane, although it appears the AAVR antibody only recognizes constructs
containing PKD1. In an attempt to identify a membrane protein AAV4 may be binding to

Figure 2.4: **AAV4 and rh32.33 are unable to bind AAVR or use AAVR for entry.** A-D) 10% SDS PAGE and viral overlay or Western blot of purified AAVR subunits. E) 4-20% SDS PAGE and viral overlay or Western blot of WT or AAVR KO A549, HeLa, or Huh7 cell lysates. F) NIH/3T3 cells stably expressing AAVR-flag or AAVRdeltaCtail transduced with $10^5$ viral genomes per cell AAVrh32.33, AAV4, or AAV2 CMV.Luciferase.SVPA. G) Western blot demonstrating AAVR expression in stably transduced NIH/3T3 cells. Data presented are mean +/- SEM of 3 independent experiments.
and using as a receptor, we did a viral overlay assay on membrane preparations from three different WT and AAVR KO paired cell lines. AAV4 does appear to bind to two proteins smaller than AAVR, of about 85 and 40 kDa in all cell lysates tested [Fig. 2.4.E]. Although we observe binding of AAV4 to something in these membrane preparations, this may or may not be a protein receptor, as these denaturing gels may alter a conformational epitope that AAV4 and AAVrh32.33 bind to. These observations are confirmatory for AAV2 to demonstrate a direct binding event to AAVR [Fig. 2.4.E] (93, 103) and are consistent with our findings from AAV4 and AAVrh32.33 binding [Fig. 2.3.B] and transduction [Fig. 2.3.C] experiments for which AAVR binding cannot be demonstrated.

**AAV4 and AAVrh32.33 are unable to use AAVR as an entry receptor.**

In addition to determining whether AAV4 and AAVrh32.33 can bind AAVR, we wanted to test whether AAV4 and AAVrh32.33 are able to use AAVR as a receptor by re-introducing AAVR into a non-permissive cell line. To do this we stably re-introduced full-length AAVR via a lentiviral vector into non-permissive NIH/3T3 cells. AAVR was able to rescue AAV2 transduction as expected, but not AAV4 or AAVrh32.33 [Fig. 2.4.F], despite high levels of AAVR expression as determined by Western blot [Fig. 2.4.G]. An inability to rescue transduction upon overexpression demonstrates that AAVR expression is not sufficient for entry of AAV4 or AAVrh32.33 in these cell lines.
AAVR is not required for AAVrh32.33 transduction in vivo.

AAvr KO and WT control mice were injected intravenously via retro-orbital injection with $10^{11}$ genome copies (GC) per mouse of AAV8, AAVAnc80, or AAVrh32.33 expressing eGFP.T2a.Luciferase. As expected, at day 7 post injection, AAVrh32.33 showed similar levels of luciferase expression in WT and KO mice [Fig. 2.5.A]. High levels of transduction were observed in WT mice for AAV8 [Fig. 2.5.B] and AAVAnc80 [Fig. 2.5.C], but background levels were observed in the Aavr KO mice. Similar levels of AAVrh32.33 transduction are observed in WT and KO mice 21 days post injection as well [Fig. 2.5.D]. Interestingly, at 21 days post-injection we begin to see luciferase expression in Aavr KO mice transduced with AAV8 [Fig. 2.5.E] suggesting that, when forced, AAV8 can undergo AAVR independent entry in vivo although with a delayed onset compared to when AAVR is present. Anc80, which also uses AAVR [Fig. 2.4.C],
did not demonstrate such a delayed AAVR independent transduction at the timepoints tested [Fig. 2.5.F]. AAVrh32.33 showed a distinctly wider biodistribution pattern based on in vivo bioluminescence imaging [Fig. 2.6.A] compared to AAV8 or AAVAnc80, which

Figure 2.6: Bioluminescence demonstrates altered biodistribution upon AAVR independent entry. Bioluminescence images demonstrating biodistribution of AAVrh32.33 (A,E), AAV8 (B,F), AAVAnc80 (C,G), or uninjected control (D,H) WT and AAVR KO mice 7 (A-C) or 21 (D-F) days post-injection.
as previously observed, is dominated by liver [Fig. 2.6.B,C]. Interestingly, AAV8 transduction in KO mice 21 days post injection had altered biodistribution compared to WT [Fig 2.6.E]. These data demonstrate that an alternate AAV entry pathway exists in vivo which AAVR independent serotypes such as AAVrh32.33, and, to some extent, AAVR dependent serotypes such as AAV8 can use for entry in vivo.

IV: Discussion:
Recent development and implementation of genome-scale genetic knockout screens have allowed identification of a multi-serotype AAV protein receptor (141), which previous methods were unable to define (140). Our data suggests that there are at least two predominant entry mechanisms within the primate Dependoviridae, namely a canonical entry pathway by which most AAV serotypes enter cells and which requires AAVR, as well as an alternate, AAVR-independent pathway used by AAV4 and AAVrh32.33. Recently, Pillay et al. (103) demonstrated that the most evolutionary distinct serotype, AAV5, primarily uses a different domain of AAVR (PKD1) than the other tested naturally isolated serotypes (PKD2). From the AAV capsid lineage, it appears that two distinct branches of AAV capsids have emerged. One branch is entirely AAVR independent, uses a currently unidentified receptor, and is composed of AAV4 and AAVrh32.33. The other distinct branch is composed of all serotypes whose putative evolutionary ancestor is AAVAnc80, and who have evolved to predominantly use PKD2 of AAVR for binding and efficient transduction. It is curious that these two monkey isolates have evolved to be AAVR independent, given the high level of conservation between human AAVR and AAVR of all non-human primates. Human and
rhesus AAVR share 98% sequence homology, suggesting that the evolution of AAVR independent serotypes may have arisen to allow unique tissue specificity to these AAVs, rather than having co-evolved with their host. Interestingly, although AAVAnc80 and AAV5 are also highly divergent from other naturally circulating serotypes, we found they are both dependent on AAVR. Investigating the sequence space occupied by the most common ancestor of the AAV4 and AAVrh32.33 branch and AAV5 or AAVAnc80 may provide insight into what dictates receptor usage by different AAV serotypes.

Since we were able to observe a low level of transduction by some serotypes both in KO cells in vitro (AAV6.2, AAV3, AAV2) and in KO mice in vivo (AAV8), it is possible that these serotypes are also able to use an alternate entry pathway. It remains to be determined if these serotypes could be using the same alternate receptor as AAV4 and AAVrh32.33. Additionally, peptide insertion mutants AAV7M8 and AAV8BPV2 are highly dependent on AAVR, demonstrating that peptide insertion does not change the requirement for AAVR. Since peptide insertions do not alter AAVR usage, we hypothesize that peptide insertion may alter another aspect of the entry pathway, such as (glycan) attachment.

Similar levels of binding and transduction by AAV4 and AAVrh32.33 in overexpression and AAVR KO cells demonstrate that AAVR does not play a role in AAV4 or AAVrh32.33 attachment. Due to the high sequence similarity of AAV4 and AAVrh32.33, it may be expected that they would use the same glycan attachment factor. However, we demonstrated that neither sialic acid nor heparan sulfate appear to play a dominant
role in attachment of AAVrh32.33. Alternatively, AAVrh32.33 may have redundant glycan usage. There is currently no predominant glycan known that AAVrh32.33 uses for attachment. A striking observation was the similar level of AAV2 binding in AAVR overexpression and AAVR KO cells. This is in contrast to previously published data that suggests a role for AAVR at the plasma membrane (93). In these experiments, cells incubated on ice with either soluble AAVR (ectodomain without transmembrane domain) or an anti-AAVR antibody showed decreased AAV2 transduction in a dose-dependent manner (93). However, these experiments did not directly assess AAV particle binding at the cell surface and it cannot be excluded that the antibodies blocked a step after AAVR was endocytosed or that it interfered with the formation of AAVR homodimers or a multimeric receptor complex (141). Although our data suggests that the predominant role for AAVR is post-attachment, it does not preclude the possibility that AAV2 comes into contact with AAVR at the plasma membrane. It is also possible that the relative affinities of the AAV2/HSPG interaction (0.1-3.7 nm $K_d$) (142-144) vs. the AAV2/AAVR interaction (150 nm $K_d$) (93) allow high levels of AAV2 attachment to occur whether or not AAVR is present at the plasma membrane, such that the relative contribution of AAVR to AAV attachment is undetectable in this assay.

Viral overlay on purified AAVR domains confirms previously published interaction of AAV2 with AAVR [Fig. 2.4.B], yet we were unable to detect binding of AAV4 to these purified proteins [Fig. 2.4.A]. AAV4 appears to bind the same two membrane proteins in a viral overlay on three different cell types [Fig. 2], although the identity of these proteins and the significance to AAV4 entry remains to be determined.
Although stable over-expression of AAVR in non-permissive NIH/3T3 cells is able to rescue AAV2 transduction, it is unable to rescue AAV4 or AAVrh32.33, suggesting that AAV4 and AAVrh32.33 use a different protein entry receptor. Since NIH/3T3 are a mouse derived cell line, it is possible that other cellular co-factors are required for AAVR usage by AAV4 and AAVrh32.33, and that these co-factors from other species are unable to interact with human AAVR. However, since AAVR is neither necessary nor sufficient for transduction of AAV independent serotypes, we hypothesize that there is an entirely separate protein receptor used by these serotypes. What this receptor is and whether these two serotypes use the same receptor for entry is currently unknown.

Our study demonstrates that AAV4 and AAVrh32.33 may be attractive for use where AAVR is rate limiting. For example, although AAVR expression is fairly ubiquitous, a large amount of vector injected intravenously gets trapped in the liver. This is in stark contrast to the wide biodistribution observed from intravenous injection of AAVrh32.33. Such evenly distributed transgene expression suggests that the entry receptor required for AAVrh32.33 entry is expressed in a wide range of tissues. Interestingly, 21 days after injection we observe low levels of luciferase expression in the AAV8 injected Aavr KO mice, although with altered distribution. AAV8 injected Aavr KO mice seem to have liver de-targeted transduction. The observed distribution pattern is similar to what has been reported for intravenous injection of AAV2 R484E; R585E (77), suggesting that residues in this region which has long been thought to play a role in receptor binding, may influence not only heparan sulfate binding, but also usage of a secondary receptor.
It is undoubtedly of interest to identify AAVR binding domain(s) on the AAV capsid in order to develop tissue specific novel AAV vectors.

The data presented here demonstrate that AAVR is the major determinant for efficient entry of most, but not all, known serotypes, and further demonstrates the immense complexity underlying the AAV entry pathway. By elucidating AAV entry mechanisms we aim to further inform targeting and delivery aspects of AAV-based gene therapies.

V: Materials and Methods:

Phylogeny Generation

To generate the phylogeny, fifteen representative isolates of AAV were chosen, including one avian AAV for use as an outgroup. Amino acid sequences of the VP1 proteins from each of these strains were aligned by ClustalOmega as implemented on the EMBL-EBI webserver (145, 146). A maximum-likelihood phylogeny was constructed using PhyML 3.0 using the LG+I+G+F substitution model and NNI tree improvements (147). The phylogeny was rendered by letting AAAV isolate VR-865 act as an outgroup and rooting on its branch.

Cell lines

All cell lines were maintained in Dulbecco's modified Eagle's minimal medium DMEM (Corning) supplemented with 10% FBS (GE Healthcare) and 100 IU/mL penicillin/streptomycin (Corning) in a humidified incubator with 5%CO₂ at 37°C. Parental
cell lines were obtained from American Type Culture Collection (ATCC, Manassa, VA), and AAVR KO cell lines were generated as previously described (93).

**AAV production and purification**

All vectors were produced, purified, and titrated by the MEEI/SERI Gene Transfer Vector Core (http://vector.meei.harvard.edu). Large scale vector preparations were generated by polyethylenimine (Polysciences, Cat#24765-2) triple transfection of pHelp, pAAVector2[Cap], and pCMV.Luciferase.SVPA, pCMV.eGFP.T2A.Luciferase, or pCMV.eGFP.WPRE.bGH transgenes in a 2:1:1 ratio. 520 µg total DNA was transfected in ten-layer hyperflasks using a PEI Max:DNA ratio of 1.375:1 (w/w). 3 days after transfection, vectors were concentrated by tangential flow filtration and purified by iodixanol gradient ultracentrifugation as previously described (148).

**AAV genome titration**

DNase1-resistant viral genomes were quantified by TaqMan qPCR (ThermoFisher, Cat# 4304449) using primer/probe set detecting CMV promoter. Vector purity was assessed by SDS-PAGE electrophoresis.

**AAV transduction**

All luciferase transduction assays excluding those done for the binding assay in Fig. 2.3 were done by seeding 10,000 cells per well in Poly-L-lysine (Sigma Aldrich Cat# P4707) coated black-bottom 96 well plates overnight. All flow cytometric transduction assays were done by seeding 1x10^5 cells per well of a 12 well plate overnight. When indicated,
cells were pre-incubated with 200 pfu/cell of WT hAd5 (University of Pennsylvania Vector core) in D10 for two hours, then hAd5 containing medium was removed prior to transduction. Cells were transduced with AAV at 1x10^4 VG/cell in serum-free DMEM for 1h at 37°C, then D10 was added and transduction levels were analyzed either by luciferase assay or flow cytometry 48h post-transduction.

**Luciferase assay**

2 days post-transduction, cell culture medium was removed and cells were lysed in 20 µL per well of 1x Reporter Lysis Buffer (Promega, Cat#), then frozen at -80°C. After thaw, ffLuc expression was measured in Relative Light Units/s on a Synergy H1 Hybrid Multi-Mode Microplate reader using 100 µL luciferin buffer [200 mM Tris pH 8, 10 mM MgCl2, 300 µM ATP, 1x Firefly Luciferase signal enhancer (Thermo Cat#16180), and 150µg/mL D-Luciferin].

**Flow cytometry**

Cells were harvested for flow cytometry analysis using PBS without Ca^{2+} and Mg^{2+}, supplemented with 5 mM EDTA at 37°C for ten minutes, then fixed in 4% paraformaldehyde for 20 minutes at room temperature. All flow cytometric analysis was done at the Massachusetts General Hospital Flow Cytometry Core (Simches Research Building) using an Amnis ImageStream mkII Imaging Flow Cytometer. Live cells were distinguished by propidium iodide staining. GFP positive cells were counted and mean fluorescence intensity was quantified using FloJo v8.8.6 software.
**Fluorescent imaging**

Live cells were imaged two days post-transduction. GFP expression in transduced cells was imaged using an EVOS FL Cell Imaging System (ThermoFisher) using 10x objective and 50% GFP intensity. Images were analyzed using ImageJ.

**Viral binding assay**

Black-bottom 96 well plates were coated with Poly-L-lysine (Sigma Aldrich Cat# P4707) by incubation at 37°C for 30min. HEK293 overexpression or HEK293 AAVR KO cells were plated on Poly-L-lysine coated plates at 100,000 cells per well overnight. Cells were incubated with 50 mU/mL Neuraminidase from Vibrio cholera Type III (Sigma Aldrich, Cat#N7885) in serum-free DMEM for 2h or untreated wells were incubated at 37°C and 5% CO₂ with serum-free DMEM alone. Vectors were either pre-incubated in serum-free DMEM or serum-free DMEM supplemented with 10µg/mL soluble heparin (Sigma, Cat#H3149) for 1h at 37°C then pre-chilled on ice for 10 minutes prior to addition to cells. After neuraminidase incubation, cells were placed on ice for 10 minutes, then 10³ VG per well pre-chilled vector (with or without heparin pre-incubation) were added in a total volume of 50 µL per well. Vectors were allowed to bind cells on ice on an orbital shaker platform for 1h. Following binding, cells were washed 3x with ice-cold PBS with Mg²⁺ and Ca²⁺ then either 50 µL PBS was added and cells were frozen immediately ("Binding" Fig. 2.3), or 200 µL pre-warmed D10 was added and cells were cultured for 2 days prior to luciferase assay ("Transduction" Fig. 2.3). Binding assay plates underwent 3 freeze-thaw cycles, prior to resuspension and viral genome quantification by qPCR as described above using CMV primer/probe.
Western blot and antibodies

Whole cell western blot samples were prepared by lysing $1 \times 10^5$ cells per sample in 0.2% Triton-X and 10U/ml DNase1 (New England Biolabs, Cat#M0303) in 1xDNaseI buffer for 30 minutes on ice. After 30 minutes samples were adjusted to contain 62.5mM Tris (pH 7), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 100mM DTT, and 0.001% bromophenol blue. Samples were boiled at 100°C for 2 minutes prior to running on a NuPage 4-12% Bis-Tris Gel (ThermoFisher) and transfer to PVDF membrane. Membranes were blocked at room temperature for 45 minutes using 5% Blotting Grade Non Fat Dry Milk in PBST (0.02%). Primary antibodies were incubated overnight in 0.05% block. Antibodies used are as follows: mouse polyclonal anti-KIAA0319L (Novus Biologicals, Cat# H00079932-B01) at 1:2,000 dilution, mouse monoclonal anti-Actin (ThermoFisher, Cat# MA1-744) at 1:10,000, sheep anti-mouse IgG, HRP conjugate (GE Healthcare, Cat#GENXA931) at 1:2,000.

Viral Overlay Assay

Individual His-tagged AAVR domains were expressed and purified as previously described (103) and recombinant proteins were loaded onto 10% or 6% SDS-PAGE gels at 0.5 µg/lane. Cellular membrane proteins were isolated using detergent-based cellular fractionation (Cell Signaling #9038). Viral overlay assay performed as previously described (103). A20 in-tact capsid antibody (American Research Products, Inc. #03-61055) was used for AAV2 viral overlay, and ADK4 in-tact capsid antibody (American Research Products, Inc. 03-651147) was used for AAV4 viral overlay.
Lentiviral production

Lentiviral constructs encoding AAVR-flag and AAVRΔC-tail have been previously described (93). Lentivirus was produced from HEK293T cells (ATCC, Manassa, VA), by transient transfection using PolyJet *In Vitro* DNA Transfection Reagent (SignaGen, Cat#SL100688) using manufacturer’s protocol for lentiviral production. HEK293T cells were seeded overnight at 4x10⁶ cells per 10cm dish. 1h prior to transfection, medium was changed to fresh pre-warmed D10, followed by transfection of psPAX2, pLenti-CMV-AAVR-flag-Puro or pLenti-CMV- AAVRΔC-tail -Puro, and pCMV-VSV-G at a 10:10:1 ratio. Medium was changed to fresh D10 6 hours after transfection, and supernatant virus was harvested 48h later, clarified by centrifugation at 2,000 RPM for 5min in Sorvall tabletop centrifuge, and filtered through a 0.45 micron filter.

Generation of stable cell lines

NIH/3T3 cells (ATCC, Manassa, VA), were seeded at 1x10⁶ cells per well of a 6 well plate the night prior to transduction. Cells were transduced by spinfection for 30min at 25°C and 2,500 RPM in tabletop using 1mL per well of supernatant lentivirus in the presence of 10 μg/μL Polybrene (ThermoFisher Scientific, Cat#TR1003G). Medium was changed to fresh D10 following spinfection, and one day later stably transduced cells were selected using 5 μg/μL puromycin (Sigma Aldrich, Cat#P9620) for 2 days.

Ethics statement

All animal studies were carried out under conditions approved by the Stanford University Institutional Animal Care and Use Committee and mice were housed in a
facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Experimental protocols were approved under Stanford IACUC APLAC protocol #28856. This protocol adheres to the guidelines set by the Public Health Service Policy on Humane Care and Use of Laboratory Animals (2015) and the Guide for the Care and Use of Laboratory Animals, 8th edition (2011).

Animal studies

Aavr KO mice carrying a 1bp deletion (cccgcttc-gggtttgccccagga where - marks the deletion) resulting in a frameshift mutation in Aavr (93) or WT cagemate mice were intravenously injected with 1x10^{11} VG/mouse via retro-orbital route. Bioluminescence using the whole mouse as region of interest was quantified 7 and 21 days post injection. Mice were anesthetized with 2% isofluorane and oxygen. 3.3 ug D-luciferin substrate per mouse was injected intraperitoneally, and 10 minutes after injection bioluminescent images were taken using an IVIS 100 cryogenically cooled charge-coupling device camera (Xenogen, Alameda, CA). Bioluminescence was recorded, at 1, 10, 60, and 100 seconds, with visual output representing average radiance in photons emitted/second/cm^2 as a false color image where the maximum is red and minimum is dark blue. The region of interest for radiance quantification (photons/sec/cm2/radian) was designated as the whole mouse.

VI: Acknowledgements

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Chapter 3:

Capsid and AAVR variation influence AAVR usage and transduction efficiency
I: Abstract:

The Adeno-associated virus subfamily of parvoviruses has a high amount of variability within the capsid gene, leading to AAV vectors with unique properties such as altered tissue targeting, specific cellular glycan binding, or receptor usage. While many glycan-binding motifs have been mapped on a variety of AAV capsids, the structural determinants dictating AAVR usage have not been determined. We used chimeric AAV capsids from AAVR dependent and AAVR independent serotypes, as well as structural and sequence analysis to rationally design capsid mutants to functionally investigate capsid sequences that determine AAVR usage. We determined that AAVR usage is dictated by the VP3 portion of capsid, outside of hypervariable regions IV and V. Additionally, site directed mutagenesis of human AAVR was used to investigate the influence that human polymorphisms may have on AAV receptor function. Functionality of AAVR from different species used as gene therapy animal models was also investigated. We were able to determine that single amino acid substitutions observed in the human population are able to completely ablate AAVR function, and suggest a minimal motif within AAVR that likely determines AAVR binding of most AAV capsids. Our studies aim to further characterize structural determinants of AAVR usage both on capsid and AAVR, to better implement rational design of AAV-based gene therapies and inform gene therapy animal model choices.

II: Introduction

The general capsid structure of AAV is similar to that of most parvoviruses. AAV is a non-enveloped icosahedral virion with T=1 symmetry, composed of three capsid
proteins of different lengths, VP1, VP2, and VP3 that have an identical c-terminal portion. These three capsid proteins are incorporated into the virion at approximately a 1:1:10 ration of VP1, VP2, and VP3, respectively (149-151). Within the capsid monomer there is an 8-stranded anti-parallel β-barrel core and α helix. These general structural characteristics are conserved within the parvovirus family. Between the beta barrels, large loops compose most of the capsid surface and dictate serotype specific differences in the capsid. Many AAV serotype structures have been resolved either through x-ray crystallography (69, 152-158) or cryo-EM (159), all of which show protrusions at the three-fold axis of symmetry, a depression at the two-fold axis, and a cylindrical pore at the five-fold axis of symmetry with a depression surrounding it.

The AAV VP1 region is unique in its containment of a parvoviral phospholipase A2 (pvPLA2) domain which exhibits phospholipase activity when expressed alone as a recombinant protein from bacteria (57). This PLA2 domain is thought to be internalized in the native capsid conformation and then extruded from the capsid to allow endosomal escape (94, 160). The predicted structure of the AAV1 VP unique region has been modeled using comparative modeling to the known structure of bovine pancreatic PLA$_2$ (161), but it has never been visualized in the crystal structure of a complete virion, either because it is disordered or because the low abundance of VP1 relative to VP3 proteins (a 1:10 ratio) makes orienting this portion of the capsid impossible with current structural imaging techniques.
At the ends of the beta barrel loops exist hypervariable regions (VR-I through VR-IX), designated by sequence conservation between AAV2 and highly divergent AAV4 (152). These hypervariable regions in some serotypes contain large insertions or deletions and have been implicated in multiple biological properties of different serotypes such as attachment factor engagement, transduction efficiency, and antigenicity. Importantly, it has been shown that these regions as well as the VP1/2 region can be swapped between serotypes to study the function of these domains. For example, two different groups have attributed liver transduction to hypervariable regions of AAV8, specifically VR VII and IX, as engraftment of these domains onto the AAV2 capsid increases liver targeting of AAV2 (162, 163). AAV2 transduction was also decreased in liver and increased specifically in cardiac and skeletal muscle by inserting a portion of hypervariable domain VIII from AAV8 (164), a vector which is under further pre-clinical development for genetic diseases of the muscle (165). Additionally, chimeric capsids containing VR swaps between rh32.33 and AAV8 identify regions of the VP3 portion of capsid that are thought to contribute to CD8+ T-cell activation (166), but the mechanisms and consequences of T-cell activation to AAV capsid are still heavily debated in the field (167-169).

Aside from the large hypervariable regions, there are also smaller well characterized domains as small as a couple amino acids which dictate attachment factor usage and biological targeting. There have been multiple domains described for different capsids that dictate glycan attachment factor binding. The AAV2 heparin-sulfate binding motif has been described by multiple groups (75, 76, 158), with the minimal motif being
composed of residues R585 and R588 (76). Interestingly, mutagenesis of residues R484 and R585 from basic arginine to acidic glutamic acid residues ablate heparin binding and also decrease liver-specific targeting of the vector while retaining similar levels of transduction in other tissues such as the heart (75). Although other serotypes such as AAV3B (170) and AAV6 (69, 70) also bind to heparin sulfate within well-defined motifs, the glycan footprint does not overlap that of AAV2. Residue K531 has been demonstrated to play a major role in heparin sulfate binding of AAV6, and mutating the AAV1 residue at this position to a lysine imparts heparin binding ability as well as increasing overall transduction efficiency (171). Interestingly, the glycan receptor footprints of various AAV serotypes are all located in different areas on the three-fold protrusions, but AAV5 sialic-acid binding residues exist within the three-fold depression at the center of the three-fold axis. Capsids have been altered to contain the glycan binding motif of other serotypes such as the galactose-binding motif engrafted onto AAV2 to alter transduction efficiency and tissue targeting (172). Although these studies demonstrate the diversity and malleability of different glycan attachment factor usage, the effect of altering an entire binding motif on the structure of neighboring capsid regions and usage of their required entry factors like AAVR is unknown.

The recently identified AAVR is a type-1 transmembrane protein with its extracellular/luminal domain being composed of a MANEC (motif at N-terminus with eight-cysteines) domain followed by 5 Ig-like PKD (poly-cystic kidney disease) domains. Of these extracellular domains, only PKD1 and PKD2 have been shown to be major determinants of AAV binding, as shown by viral overlay assays and transduction assays
with rescue constructs containing truncated forms of the protein (93, 103). A c-terminal tail truncation lacking the ability to rescue transduction in AAVR knock out cells demonstrates that the c-terminal domain is also a major determinant of AAVR function (93). This domain however was shown to primarily be responsible for trafficking to the correct sub-cellular compartment, as swapping the c-terminus with the c-terminal trafficking domain from other well-characterized receptors shows that the most efficient rescue occurs when the receptor traffics far into the endosomal system, to the trans-golgi network. It is of note that the most divergent AAV serotype, AAV5, uniquely binds to and requires PKD1 for transduction, whereas all the other tested serotypes were highly dependent on PKD2 alone or a combination of PKD1 and 2 (103).

There is currently no solved structure of AAV in complex with AAVR or any of its other proposed protein receptors. There have been several structures determined which highlight AAV2 in complex with heparin (173) or a heparin polysaccharide (174) as well as an AAV2, 8, and 9 chimeric variant, AAV-DJ, in complex with a heparin polysaccharide, fondaprinux (175). The structures containing larger polysaccharides highlight that there is likely a large attachment receptor footprint on the capsid that is composed of multiple basic patches, all adding up to contribute to the affinity of AAV2 to heparin sulfate proteoglycan.

Due to the lack of known protein receptor binding sites on the AAV capsid, we have tested chimeric capsids of AAVR dependent and independent serotypes, in combination with rationally designed site-directed mutagenesis of AAV capsid and AAVR in an
attempt to define regions required for AAVR function. We have determined that the VP3 portion of capsid dictates receptor usage, and the hypervariable regions IV and V do not play a role in AAVR usage. We have identified key residues in the partially dependent AAV6.2 that appear to increase AAVR independent entry, yet have been unable to completely ablate AAVR usage in this serotype. We have additionally identified single amino acids substitutions which exist in the human population that cause a complete loss of function of AAVR for serotypes requiring PKD2 of AAVR, and these defective polymorphisms are serotype specific. We have thus narrowed down both the portions of capsid as well as AAVR that are necessary for use as a viral entry receptor in hopes that these findings will contribute to rational design of AAV vectors in the future.

III: Results:

AAVR usage is dictated by the VP3 portion of capsid, outside of hypervariable regions IV and V.

In order to identify potential AAVR binding domains on the capsid, we decided to take advantage of the ability to swap the VP1/2 region as well as the hypervariable regions of capsid. We used 6 different previously published chimeras generated between AAV8 and AAVrh32.33, two serotypes that are dependent and independent on AAVR, respectively [Fig. 3.1.A]. We used these constructs to package a CMV.luciferase transgene from HEK293 cells, and used 200 µL of virus from a crude lysate preparation to transduce Huh7 WT and AAVR KO cells, followed by luciferase assay to quantify transduction. Since intact capsid has been shown to bind recombinant AAVR, and the VP1/2 portion of capsid is thought to be internal in the native capsid, it is not surprising
that the VP1/2 domain swaps did not change AAVR usage [Fig. 3.1.B]. Swapping hypervariable regions IV and V decreased overall transduction, yet were unable to alter AAVR dependency, as all chimeras which were composed primarily of AAV8 still had a

Figure 3.1: **AAVR binding domain is located in the VP3 portion of capsid, outside of hypervariable regions IV and V.** (A) diagram of chimeric capsids tested in Huh7 AAVR KO cells. (B) Luciferase assay of Huh7 WT or AAVR KO cells 2 days post-transduction with 100 µL per well crude vector prep of indicated capsid packaging a CMV.eGFP.T2A.Luciferase transgene
roughly ten-fold decrease in transduction in the AAVR KO cells relative to WT cells [Fig. 3.1.B]. Although we were unable to identify a distinct AAVR binding motif using these chimeras, it demonstrates that AAVR usage lies within the VP3 portion of capsid and that the hypervariable region IV or V from rh32.33 engrafted onto AAV8 are not sufficient alone to allow AAVR independence.

**Residues 592 and 531 cooperate to increase AAVR independent entry**

When examining overall transduction of different AAV serotypes, we observed that AAV6.2 was fairly unique in its ability to highly transduce AAVR KO cells, while still appearing partially dependent on AAVR at high amounts of vector genome/cell [Fig. 2.2.B]. This is in stark contrast to AAV1, which is only 5 amino acids different in sequence identity than AAV6.2. Overall transduction of AAV1 is about ten-fold lower than AAV6.2, yet AAVR independent entry of AAV1 is 1,000-fold less than that of AAV6.2 [Fig. 3.2.A]. Of the 5 amino acid differences, only three are surface exposed and exist along the three-fold axis of symmetry, as highlighted in red on the AAV6 crystal structure [Fig. 3.2.B]. We used sequence alignments of AAVR dependent and independent serotypes in combination with structural characteristics to rationally design mutants neighboring these three residues that may cause AAV1 and/or AAV6 to become more AAVR independent. Most mutations in AAV1 or AAV6.2 towards the AAVR independent identity at that position had little effect on AAVR independent entry [Fig. 3.2.C]. However, we were able to identify two residues, K531 and T592 that increase AAVR transduction individually, and appear to have an additive effect when
assessed in combination [Fig. 3.2.D]. When observing the surface rendering of AAV6

**Figure 3.2:** Surface exposed residues suggest additive effect of distant residues in AAVR independence. (A) Transduction comparison of the highly related AAV serotypes AAV1 and AAV6.2 in Huh7 WT verses Huh7 AAVR KO cells when transduced by 100 μL per well crude vector. (B) Surface representation of surface-exposed AAV1 vs. AAV6.2 amino acid differences [highlighted in red], mapped on the AAV6 crystal structure. Highlighted residues lie near the interface of individual monomers at the three-fold axis of symmetry [individual monomers highlighted in...
Figure 3.2 (Continued): white, grey, and black]. (C) Transduction of rational-design capsid variants from the AAVR dependent toward the AAVR independent amino acid identity. (D) Partial AAVR independence mutations were combined to generate multiple capsid point mutations and Huh7 WT or AAVR KO cells were transduced with 100 µL crude vector.

trimer with the hypervariable regions IV and V highlighted in dark blue as well as the single, double, or triple point mutants generated in lighter blues [Fig. 3.3], it is clear that because the tested mutations were not sufficient to change receptor usage, the AAVR binding domain or the binding domain of the as-yet unidentified alternate receptor likely has a large binding footprint on the capsid. Due to the large distance between the two residues which increase AAVR independent entry, highlighted in red on the trimer [Fig. 3.3], it suggests that larger structural arrangements may also be required to change the receptor used by different AAV serotypes.

Figure 3.3: Surface exposed AAV residues are highly mutable without altering receptor usage. Surface rendering of AAV6 capsid crystal structure with individual monomers highlighted in white, grey, and black, plus indicated mutations of combinations of mutations highlighted in the indicated color.
Human polymorphisms in AAVR cause a complete loss of function for some AAV serotypes

Multiple AAV-based gene therapy clinical trials have been done since people started being excluded for having anti-AAV antibodies, yet there is still large patient-to-patient variability that cannot be explained. For this reason, we wanted to know whether human genetic variation in the AAV receptor could explain some of this variation. We used the GnomAD database developed at the Broad Institute to query 15,496 different human whole genome and 123,136 exome sequences for polymorphisms within AAVR. Within this dataset, we identified 539 different loss of function or missense mutations spread across the coding region of AAVR [Fig. 3.4.A]. We developed an in-vitro based assay to rescue AAVR expression via transient transfection in Huh7 AAVR KO cells using a flag-tagged AAVR construct driven from a CMV promoter. AAVR SNPs causing either a missense or truncation within PKD2 (previously shown to be required for transduction of most AAVs) were assessed for their ability to rescue AAV transduction using this assay. Anc80 was initially chosen to screen these mutants as it is the most highly AAVR dependent serotype identified thus far. Using a PKD2 deletion construct designed in our lab, we were able to confirm that Anc80 is highly dependent on PKD2, as this construct was unable to rescue Anc80 transduction [Fig. 3.4.B]. We confirmed that the c-terminal domain is also required, as a missense mutation causing an early termination codon in PKD2 (Q432Ter) was also unable to rescue Anc80 transduction. Interestingly, we identified three single amino acid substitutions, S433R, D436N [Fig. 3.4.B], and P408H [Fig. 3.4.C], which individually showed a complete loss of function of AAVR for Anc80.
This loss of function is serotype specific, as transduction of AAV5 was rescued by these

Figure 3.4: Human variation in AAVR leads to altered functionality as an AAV receptor for some AAV serotypes. (A) Histogram of human SNP frequency
Figure 3.4 (Continued):
throughout the coding region of AAVR from gnomAD database, color-coded to correspond to individual domains mapped above [MANSC: dark red, PKD1: orange, PKD2: yellow, PKD3: green, PKD4: light blue, PKD5: dark blue, Transmembrane: light purple, Cytoplasmic tail: dark purple]. Tested SNPs are shown in black, while SNPs demonstrating a loss of function as an AAV receptor are highlighted in bright red. (B) AAVR PKD2 point mutants or termination mutants transfected into Huh7 WT and Huh7 AAVR KO cells followed by transduction with 10,000 VG/cell Anc80.CMV.Luciferase.SVPA. AAVR point mutants located in either PKD1, PKD2, TM, or C-tail observed 5 or greater times in the gnomAD database transduced with either 10,000 VG/cell Anc80 (C) or AAV5 (D) CMV.Luciferase.SVPA transgene.

mutants. This suggests a specific loss of Anc80 binding within PKD2 when these residues are mutated. We tested several SNPs within PKD1 for their ability to rescue AAV5, as well as SNPs within the c-terminal trafficking domain, but all were still functional to close to wild-type levels [Fig. 3.4.C,D]. These mutants were simultaneously transfected into wild type Huh7 cells in order to assess whether any of these SNPs may cause a dominant negative effect on AAV transduction. Although there was some variability of transduction between the different constructs, none decreased transduction in wild type cells to below the level of an empty vector transduction [Fig. 3.4.D], suggesting that none of the tested SNPs are dominant negative polymorphisms. These data suggest that human variation in AAVR may cause a loss of function as an AAVR receptor in some people.

Species variation in AAVR may cause altered receptor efficacy
Expanding from these human SNP studies, we wanted to know whether species variation within AAVR may influence AAVR function. There are currently multiple different models of AAV-based gene therapy, the most commonly used being mice, and dogs, but pigs and non-human primates are also studied fairly regularly. To determine
the functionality of AAVR from these different animal models, we used flag-tagged constructs that were codon-optimized for human expression. We used the same transfection-based rescue assay as previously described, and tested the ability of human, rhesus, mouse or dog AAVR constructs to rescue AAVs that use different domains of AAVR for binding. There was no difference in transduction of any serotypes tested when these constructs were transfected into Huh7 WT cells [Fig. 3.5.A,B]. The rhesus AAVR construct was able to rescue transduction of all AAVR dependent

![Figure 3.5](image)

**Figure 3.5: Canine and murine AAVR are poorly functional in human cells.** Dose-response of flag-tagged AAVR constructs from different gene-therapy animal model species transiently transfected into Huh7 WT (A,B) or Huh7 AAVR KO (C,D) cells followed by transduction by 10,000 VG/cell Anc80 (A,C) or AAV5 (B,D) CMV.Luciferase.SVPA with hAd5 helper virus.
serotypes in AAVR knock-out cells, while having no effect on the AAVR independent rh32.33 (data not shown). In contrast, the mouse AAVR construct had a roughly ten-fold lower level of rescue compared to human and rhesus, and dog AAVR was not able to rescue transduction at all [Fig. 3.5.B]. We were able to detect expression of all constructs tested using an anti-flag antibody except for the mouse AAVR construct. It is curious that we were able to observe partial rescue with the mouse AAVR construct despite it being expressed at undetectable levels, yet we were unable to observe rescue with the dog AAVR.

AAVR overexpression can enhance transduction of a subset of AAV serotypes

While testing different AAV capsids in HEK293 WT, AAVR KO, or AAVR overexpression (rescue) cell lines, we observed that transduction of some serotypes was enhanced in AAVR overexpression cells relative to WT cells [Fig. 3.6.A]. Relative expression levels were determined using anti AAVR western blot [Fig. 3.6.B]. Our observations of which serotypes are enhanced by AAVR overexpression or not were consistent with previously published data using a CMV.eGFP transgene (93). Upon examining commonalities between capsids that were unable to be enhanced by high levels of AAVR, we noticed that many serotypes that were unable to be enhanced, such as AAV2, AAV6.2, and AAV1 have a known glycan attachment factor that plays a major role in facilitating high levels of attachment at the cell surface. Conversely, serotypes that undergo enhanced entry in the AAVR overexpression cells either have no known attachment glycan, such as AAV8, or their primary attachment glycan is the penultimate saccharide in many glycans, such as the galactose-binding AAV9 [Fig. 3.6.A].
therefore hypothesized that serotypes with a high-affinity glycan have such efficient attachment that any role AAVR may play in attachment is negligible, and that rather AAVR’s role is primarily a downstream role such as trafficking to the trans-golgi-network (93). Alternatively, recycling of AAVR to the plasma membrane may allow serotypes without a high-affinity glycan to be taken up more efficiently, and thus plays a role in both attachment and trafficking. We therefore hypothesize that AAVR may play a role in attachment only for those serotypes which do not have a high-affinity glycan.

Figure 3.6: AAVR overexpression enhances transduction of a subset of AAV serotypes. (A) Fold increase in transduction in AAVR overexpression HEK293 cells [293 Rescue] compared to WT HEK293 cells by different capsid serotypes assessed by luciferase assay. CMV.Luciferase.SVPA transgene [solid bars] or CMV.eGFP.T2A.Luciferase transgene [hashed bars]. (B) Western blot demonstrating level of AAVR expression in WT, AAVR KO, or AAVR Rescue cells.
**IV: Discussion:**

Based on our inability to alter receptor usage using hypervariable domain swap mutants [Fig. 3.1] or single, double, or triple point mutants [Fig. 3.2], it is likely that there is a large receptor footprint on the capsid that is required for AAVR binding. By definition it is unlikely that hypervariable regions may contain a binding site for a conserved entry receptor, yet it is impressive that altering such a large portion of the surface of the capsid had little to no effect on AAVR usage. The large distance between residues 592 and 531 which increase AAVR independent entry also suggest that AAVR or at least the as-yet unidentified AAVR2 may have a large binding footprint or that a larger structural rearrangement is required to change receptor usage. More recent structures of AAV2 in complex with heparin polysaccharide (173) and AAV-DJ in complex with fondaparinux (175) demonstrate that the heparin binding site likely encompasses a much larger portion of capsid than was originally defined, which may be true not only of heparin binding but of AAVR binding as well. Additionally, because different AAVs use different PKD domains within AAVR (AAV5 requires PKD 1 while most others require PKD2 (103)), there may be multiple binding modes for the AAV-AAVR interaction, potentially with different parts of capsid having different affinity to these PKD domains. It is of interest to identify AAV receptor binding sites due to the potential of rational engineering of capsid for desired targeting and gene therapy properties.

There have been multiple interesting phenotypical differences observed when altering the residues that are different between AAV1 and AAV6 that may be attributable to differences in receptor dependency (134, 171). For example, the K531 residue has
been shown to increase both overall transduction and liver-specific targeting of AAV1 and AAV6 (134). Additionally, AAV6.2 was shown to have superior transduction in both human and mouse airway epithelium compared to AAV5 (176). Interestingly, an AAV5 variant, AAV2.5T, that also has increased HAE targeting (177) contains an analogous point mutation to our 592T mutation in which we observed increased AAVR independence. This suggests that an alternate receptor may be enriched in this tissue type that is uniquely available to these partially AAVR independent serotypes.

Our studies with AAVR variants are the first to demonstrate that human and animal genetic differences in a cellular factor required for AAV entry may alter the efficiency of transduction by different serotypes. Although different expression levels were observed for different animal AAVR constructs (data not shown), it seems unlikely to be the major defining factor for efficient AAV entry since expression levels of each of these constructs did not correlate with their level of rescue. We instead hypothesize that there may either be altered AAV-binding affinity, or altered trafficking properties of these constructs due to different amino acid sequence in the binding and trafficking domains (for sequence alignment, see Appendix A). Alternatively there may be another cellular co-factor that is required to engage AAVR, but the human co-factor cannot engage AAVR from these other species. Because mice, dogs, and non-human primates have been successfully transduced with AAV and the AAVR knock-out mouse causes a loss of AAV transduction in vivo (93), it is clear that these receptors from other species can in fact function as an AAV receptor, but it is currently unclear why we can not observe efficient rescue of AAV transduction in human cell lines. Our SNP variant studies
demonstrate not only that human variation in AAVR can influence AAV transduction, but also further define the AAV binding site on AAVR and demonstrate that as little as a single amino acid change is sufficient to block binding of at least one tested serotype, Anc80 [Fig. 3.4.B, C]. Although predicted missense and loss of function variants are relatively common, about 3.8%, it is unlikely to play a major role in AAV transduction differences in patients due to the low prevalence of SNPs showing an AAVR loss of function phenotype. Within the sequenced population, SNPs for which people have been identified as homozygous either exist within domains that have been deleted while retaining AAVR function (93, 103), or were tested and shown to be functional in our rescue assay. Of those sequenced that are heterozygous, the prevalence of a loss of AAVR function within the binding domain is 0.01%, and an expected loss of function due to a frameshift or altered splice site is only 0.022%. Although this is a very small percentage of the population that may be affected by these SNPs, it is a proof-of-concept that understanding the cellular factors involved in AAV entry and the genetic variation within these factors may help dictate how targeted therapies are designed and delivered.

While it is clear that expression of AAVR is a major requirement for transduction by most AAV serotypes (93), the differential transduction of many serotypes in WT vs. AAVR overexpression HEK293 cells suggests that the level of transduction may be able to be fine-tuned in certain cell types with differential AAVR expression. Our data suggest that in the absence of a high-affinity glycan cells may be able to uptake more AAV when there is a higher level of AAVR being expressed. In fact, uptake of AAV into
the cell is thought to be a major barrier for infection, as penetration at the plasma membrane has been measured using live imaging studies and was shown to be only 13% for those viruses that undergo contact events (78). The authors of this study suggest that this may be due to a failure of interaction with a viral receptor or co-factor, which is consistent with our hypothesis. While tissue expression of AAVR appears fairly ubiquitous, it is unclear whether specific cell subsets within a particular tissue may have different AAVR expression levels. If this is the case, these cells may preferentially be targeted by a serotype that is enhanced by AAVR overexpression to lead to more specific gene therapies. Although we are only slowly beginning to understand the role AAVR plays in AAV transduction, our mechanistic data highlight the complexities that dictate cellular targeting and transduction levels of different AAV serotypes.

**Materials and Methods:**

**Cell culture**

All cell lines were maintained in Dulbecco’s modified Eagle's minimal medium DMEM (Corning) supplemented with 10% FBS (GE Healthcare) and 100 IU/mL penicillin/streptomycin (Corning) in a humidified incubator with 5% CO₂ at 37°C. All cell lines were a gift from Jan Carrette lab and were previously published (93). Cells were transfected using PolyJet *In Vitro* DNA Transfection Reagent (SignaGen, Cat#SL100688) using the standard protocol. Expression of flag-tagged AAVR constructs was determined by western blotting of whole-cell lysates using mouse anti-flag clone M2 antibody (Sigma F1804).
Plasmid constructs

AAV8 and rh32.33 chimeric constructs were previously published (166) and were a gift from James Wilson’s lab. AAV6.2 point mutants were generated by site-directed mutagenesis (Agilent QuikChange II Site-Directed Mutagenesis Kit #200524). pcDNA3.1(-).AAVR-flag was generated by addition of unique restriction sites by PCR amplification (forward primer ATCATGCGGCCGCAATGGAGAAGAGGCTGGGAGTC, reverse primer TCGATGGATCCTTACTTATCGTCGTCATCCTTGTAATCC) from pLenti.CMV.AAVR-flag.puro (93) followed by subcloning into pcDNA3.1(-) plasmid using NotI and BamHI (NEB) restriction sites. Rhesus, mouse, and dog AAVR constructs were designed by codon optimization (IDT) and flag-tagged constructs were synthesized by Genewiz in pcDNA3.1(-) plasmid. AAVR SNP variants were generated by site-directed mutagenesis of pcDNA3.1(-).AAVR-flag either in our lab or by Genewiz.

AAV production and purification

High titer AAV5 and AAVAnc80 vectors were produced, purified, and titrated by the MEEI/SERI Gene Transfer Vector Core (http://vector.meei.harvard.edu). Large scale vector preparations were generated by polyethylenimine (Polysciences, Cat#24765-2) triple transfection of pHelp, pAAVector2[Cap], and pCMV.Luciferase.SVPA, pCMV.eGFP.T2A.Luciferase, or pCMV.eGFP.WPRE.bGH transgenes in a 2:1:1 ratio. 520 µg total DNA was transfected in ten-layer hyperflasks using a PEI Max:DNA ratio of 1.375:1 (w/w). 3 days after transfection, vectors were concentrated by tangential flow filtration and purified by iodixanol gradient ultracentrifugation as previously described (148). Chimeric and point mutant viral vectors were produced on a smaller scale as
crude viral preparations by same transfection method in 10 cm cell culture plates. Three
days after transfection cells and supernatant were collected, subjected to three freeze-
thaw cycles, then crude virus preparation was clarified by centrifugation for 10 min at
10,000 RPM in a ThermoScientific FIBERLite F15-8x50cy rotor at 4°C

**AAV genome titration**

DNase1-resistant viral genomes of iodixanol purified vector preps were quantified by
TaqMan qPCR (ThermoFisher, Cat# 4304449) using primer/probe set detecting CMV
promoter. Vector purity was assessed by SDS-PAGE electrophoresis.

**AAV transduction**

All luciferase transduction assays were done by seeding 10,000 cells per well in black-
bottom 96 well plates overnight. When indicated, cells were pre-incubated with 200
pfu/cell of WT hAd5 (University of Pennsylvania Vector core) in D10 for two hours, then
hAd5 containing medium was removed prior to transduction. Cells were transduced with
either AAV at 1x10⁴ VG/cell in 50 µL serum-free DMEM (AAVR rescue experiments) for
1h at 37°C, then D10 was added to a total volume of 200 µL, or 100 µL per well of crude
virus prep (chimeric and point mutant capsid experiments) was added for 1h at 37°C,
removed, then D10 was added. Transduction levels were analyzed by luciferase assay
48h post-transduction.
Luciferase assays

2 days post-transduction, cell culture medium was removed and cells were lysed in 20 µL per well of 1x Reporter Lysis Buffer (Promega, Cat#), then frozen at -80°C. After thaw, ffLuc expression was measured in Relative Light Units/s on a Synergy H1 Hybrid Multi-Mode Microplate reader using 100 µL luciferin buffer [200 mM Tris pH 8, 10 mM MgCl2, 300 µM ATP, 1x Firefly Luciferase signal enhancer (Thermo Cat#16180), and 150µg/mL D-Luciferin].

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Chapter 4:
Identification of alternate AAV entry pathway factors by genome-wide CRISPR-Cas9 knock-out screen
I: Abstract:

Adeno-associated virus is a highly promising vector for therapeutic gene transfer, yet cellular determinants of AAV entry are poorly understood. We previously identified a subset of AAVs that do not use the canonical AAV receptor, AAVR, leading us to question what other cellular factors are involved in the AAV entry pathway of different serotypes. We employed a genome-wide CRISPR/Cas9 screen and identified a second highly conserved AAV entry factor, GPR108, required for all AAV variants tested aside from the most highly divergent capsid, AAV5. We additionally identified a complex comprised of NEU1 and CTSA required specifically by AAVR independent serotypes. NEU1 or CTSA knockout (KO) in MEF cells demonstrate a 10 to 100-fold decrease in AAV4 or rh32.33 transduction, respectively. Chemical inhibitors demonstrate a requirement of NEU1 enzymatic activity for entry of AAVR independent capsids, but show no effect on AAVR dependent capsids, including those who use sialic acid as an attachment factor. Cellular binding assays demonstrate no difference in cellular attachment of AAV4 or rh32.33 in NEU1 KO or CTSA KO cells compared to WT cells, suggesting a role in a bona-fide receptor complex. GPR108 knockout in multiple human cell lines demonstrate a 10 to 1,000-fold decrease in transduction of all serotypes, which can be rescued by GPR108 cDNA. Cellular binding assays demonstrate similar levels of attachment for all serotypes tested in GPR108 KO, AAVR KO, or AAVR/GPR108 double KO cell lines, relative to WT, suggesting a major role in AAV entry that is post-attachment. Importantly, GPR108 KO in the mouse hepa cell line causes roughly a 100-fold decrease in transduction by hepa-permissive AAV variants, and mouse GPR108 is functional to rescue a GPR108 KO in human Huh7 cells,
demonstrating a high conservation of this AAV entry factor across species. Chimeric AAV capsids generated from GPR108-dependent AAV2 and GPR108-independent AAV5 demonstrate that GPR108 usage is determined by the VP1u portion of capsid, suggesting a conformational change must occur in capsid before GPR108 engagement, and highlighting a potential role for GPR108 in endosomal escape, as VP1u contains a highly conserved phospholipase domain. We therefore propose a novel entry mechanism by which most AAVs require both of the highly conserved entry factors AAVR and GPR108, yet AAV5 uses an as-yet unidentified cellular factor in place of GPR108, and AAV4 and rh32.33 use a minimal complex of NEU1/CTSA in place of AAVR. Our studies have significant implications for vector design and implementation of these AAV-based gene therapies.

II: Introduction:

Previous methodologies have been insufficient to identify major AAV entry factors and characterize subfamily-wide receptor and entry factor requirements. Previous studies have primarily focused on cDNA overexpression in poorly permissive cell lines to identify factors that increase transduction of a particular serotype (81, 85, 86), most often AAV2 (79, 80, 82). These studies have identified several proteins that increase AAV transduction, yet the mechanism by which they influence transduction has been poorly characterized aside from often demonstrating increased attachment at the cell surface upon overexpression (83). There is a disconnect in the data however, as knock-down and knock-out studies of these factors often do not show a major defect in AAV transduction, and thus can not be defined as a required entry receptor. We pose that
these previously reported factors act as protein attachment factors, and that the major AAV receptors, co-receptors, and potentially other entry factors have yet to be identified and characterized.

Several groups have also undertaken siRNA library screening in an attempt to identify either AAV entry factors or restriction factors. This method however is often insufficient to identify major viral receptors due to the high efficiency at which these receptors function, such that virtually none of the protein can be expressed in order to observe a decrease in transduction. Such low expression levels are often not achieved by siRNA knock-down, and these screens are subject to off-target effects leading to false positives, and insufficient knock-down levels leading to false negatives. While several groups have reported identification of potential AAV restriction factors via siRNA screen (178-180), there is little overlap in the undertaken screens and the mechanism behind restriction particularly in the context of different AAV serotypes has yet to be described, suggesting that these screens may have been subject to the described false positive and false negative problems.

In the past several years, novel methods have led to a drastic increase in the identification of major virus entry receptors, co-receptors, entry factors, and restriction factors. Studies beginning in the late 2000’s to early 2010’s used variant of the KBM7 cell line that is haploid for every chromosome but chromosome 8 (181). This cell line, Hap1, was then transduced with a lentiviral vector that inserted a strong splice acceptor site, effectively causing any gene with an integration site to become inactivated (181).
The cells could be subjected to a strong selective pressure such as infection with a cytotoxic virus, then inactivated genes were mapped by lentiviral integration site deep-sequencing to identify the required entry factors. This method has led to the identification of receptors for several important viruses or virus family such as the identification of the Ebola virus receptor (182). Recently, this method was used to identify a previously uncharacterized type-I transmembrane protein, KIAA0319L, shown to be a canonical AAV receptor (93). Knock-out of this protein in multiple cell lines demonstrates an almost complete loss of transduction of all serotypes tested in this initial publication. As most AAV studies to date have identified different glycans and proteins involved in transduction level of a variety of serotypes, it was thought that there was no canonical AAV receptor and that each serotype used a different set of receptors/co-receptors. The identification of this important AAV receptor using haploid screening highlights the power of this type of methodology in studying virus-host cell interactions and entry requirements.

While haploid screening is an extremely powerful method, screens are limited to those that can be carried out in this single cell line, Hap1. Recent development of the CRISPR/Cas9 system has led to the ability to undertake genetic screens in any desired cell line that can be transduced with a pseudotyped lentiviral vector (183-187). The most popular of these CRISPR/Cas9 screening methods has been developed for both human and mouse cells, and includes sgRNAs spanning the entire genome and also contains sgRNAs agains micro RNAs and has been optimized for high titer vector production (188). Screening is done by transducing the cell line of interest with Cas9, followed by a
lentiviral library containing multiple sgRNAs targeting every gene in the genome. After selection, deep sequencing of the sgRNA sequence identifies enrichment of known sgRNA sequences (compared to the unselected control population) against a particular gene to determine the significant hits. To minimize false negative and false positive results from off-target effects and inefficient sgRNAs, an aggregate measurement is determined to take into account relative fold enrichment of multiple sgRNAs per gene, a term called Robust Rank Aggregation (RRA) (189) initially used for analysis of siRNA screens. This CRISPR screening method has identified multiple viral entry factors (190-198), and the utility and application of these screens for investigation of virus biology has been reviewed in detail in (199). CRISPR screens have been demonstrated to have fewer false positive and false negative results compared to siRNA and shRNA screens (200), and comparison of Haploid screening verses CRISPR screening has demonstrated the ability to identify the same entry receptors using either method (191).

We have employed a CRISPR screen to identify AAV entry factors in an attempt to better understand the basic biology of AAV and in an attempt to explain and better translate AAV-based gene therapies. We have recently described a subset of AAV serotypes that do not use the canonical AAV receptor, AAVR (201). These serotypes do not require AAVR in vitro or in vivo, and are unable to use or bind to AAVR. We therefore wanted to determine what alternate entry receptor these serotypes use, if it is the same alternate receptor within this subgroup, and if there are any entry factors in common with the canonical entry pathway. Testing of a variety of cell lines demonstrated high-levels of transduction in Huh7 cells sufficient to carry out an entry
factor screen using an AAVR independent serotype, rh32.33. Using the human GeCKO V2 library we have identified and characterized several factors involved in this alternate AAV entry pathway, as well as identification of a common entry factor used by all tested AAV serotypes but AAV5.

This work presents two novel entry factors, Neuraminidase 1 (NEU1) and Cathepsin A (CTSA) required specifically by AAVR independent serotypes, rh32.33 and AAV4. These two proteins exist in a lysosomal multienzyme complex (LMC) together with beta-galactosidase (202, 203), and CTSA is required for stability of NEU1 and for maintaining the catalytically active conformation of the enzyme (204, 205). Small molecule experiments demonstrate that enzymatic activity of NEU1 is required for its entry factor function for these AAV serotypes. It is not surprising that NEU1 (Neuraminidase 1), a factor involved in cellular glycan biology, was implicated in AAV entry; yet we demonstrate that this factor is not involved in alteration of attachment at the cell surface, suggesting a role in a bona-fide receptor complex.

We additionally identify another canonical entry factor, GPR108, a factor also identified in the AAV2-based haploid screen that identified AAVR (93). Using CRISPR knock-out in multiple cell lines and cDNA rescue experiments, we demonstrate that this protein is required for entry of all AAV serotypes aside from the most highly divergent serotype, AAV5. We demonstrate that GPR108 is not required for cellular attachment, and likely plays a role in endosomal escape of the virus, as GPR108 usage is dictated by the VP1 portion of capsid containing the phospholipase domain. We demonstrate that usage of
this entry factor is conserved in mouse, and that mouse GPR108 is highly functional in human cell lines. Investigation into the mechanism by which this uncharacterized protein functions in AAV entry biology will help further our understanding of AAV biology and development of novel AAV gene therapy vectors.

III: Results:

Entry screen identifies rh32.33 entry factors

We hypothesized that since rh32.33 and AAV4 do not require and can not use AAVR for entry (201), there may be many host cell factors used for rh32.33 and AAV4 entry that were not identified in the AAV2 haploid entry screen. We thus designed a CRISPR-based entry screen to identify cellular entry factors required for the alternate AAV entry pathway. A two-vector lentiviral system introduces Cas9 in a single vector to the cell line of interest, followed by a library of sgRNAs and miRNAs spanning the entire human genome [Fig. 4.1.A] (185). Huh7 AAVR KO cells were used for this screen to assure that any possible redundancy with AAVR-dependent entry would not cause false negatives in the screen. Multiple rounds of transduction of lentiCRISPR mutagenized cells transduced with a rh32.33.CMV.eGFP.WPRE vector and FACS sorting followed by Illumina deep sequencing of sgRNA prevalence are used to identify cellular factors involved in either AAV restriction or AAV entry [Fig.4.1.B].
30 million cells mutagenized with each half of the lentiCRISPR library (V2A cells and V2B cells) (188) were transduced with a high MOI of rh32.33.CMV.eGPF.WPRE. The

Figure 4.1: rh32.33 entry screen design. (A) Two-vector lentiviral GeCKO system. Cells are transduced with vector 1 [lentiCas9-Blast] followed by blasticidin selection, for stable expression of Cas9. Cas9 are then transduced with vector 2 [lentiGuide-Puro] in a library format containing sgRNAs targeting the entire human genome to generate a cell line knock-out library. (B) Huh7 AAVR KO cells undergo lentiCRISPR mutagenesis using vectors described in A. Cells are subjected to multiple rounds of high MOI transduction and sgRNA deep sequencing to determine gene deletions enriched in GFP+ or GFP- cells, identifying factors potentially involved in rh32.33 restriction or entry, respectively.
cells with the highest ~15% mean fluorescence intensity (MFI) were selected and sgRNA prevalence was deep sequenced to identify cellular factors that may be restricting AAV entry or gene expression [Fig. 4.2.A,B]. Cells that were GFP- [Fig. 4.2.A,B] were selected, split in half and either deep sequenced or subjected to another round of transduction. These cells were transduced at the same MOI and GFP- cells

Figure 4.2: **FACS selection of rh32.33 entry screen.** Gating strategy for FACS selection of GFP+ and GFP- cells in each of two halves [V2A and V2B, (A,B)] of the GeCKO library. Gating strategy for FACS selection of GFP- cells expanded and subjected to a second round of high MOI transduction for each half (C,D) of the GeCKO library.
were sorted and sequenced for further enrichment of rh32.33 entry factors [Fig. 4.2.C,D]. The second round of transduction, although done at the same MOI, had a higher percentage of cells that remained GFP- [Fig. 4.2.C,D] relative to the first round of transduction [Fig. 4.2.A,B], suggesting that selection enriched for deleted genes.

![Image of gel electrophoresis](image)

**Figure 4.3:** Sample preparation and NGS reads from selected or unselected cell populations. (A) A two-step nested PCR strategy amplifies sgRNA’s for sequencing from unselected (ctrl) or first round GFP+ or GFP- cell populations, adding a unique sample barcode and Illumina adaptors in the NGS amplicon. (B) Raw and mapped reads from Illumina deep sequencing of gel-purified amplicons from each control or selection condition [V2A and V2B combined] used for downstream gene enrichment analysis.

<table>
<thead>
<tr>
<th>Label</th>
<th>Reads</th>
<th>Mapped</th>
<th>Percentage</th>
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<tbody>
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<td>1 control</td>
<td>20898001</td>
<td>12072581</td>
<td>0.58</td>
</tr>
<tr>
<td>2 minus</td>
<td>9308273</td>
<td>5227205</td>
<td>0.56</td>
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<tr>
<td>3 plus</td>
<td>10004581</td>
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<td>4 minus2</td>
<td>7267225</td>
<td>3726076</td>
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Table 1: Summary of comparisons
required for rh32.33 entry. The genomic DNA was extracted from the different selected cell populations, and the sgRNAs were amplified by a two-step nested-PCR strategy that added sample-specific barcodes and Illumina adaptors (206) for deep sequencing [Fig. 4.3.A]. The samples were multiplexed and sequenced, followed by combining the V2A and V2B samples to analyze the sgRNA prevalence in the full library, and reads were mapped back to known sequences within the lentiCRISPR V2 library. Each selection condition produced greater than 7 million total reads and greater than 3.7 million reads mapped perfectly to the known input sgRNA sequence [Fig. 4.3.B], enough to maintain greater than 300-fold coverage of the sgRNA library.

**Identification of potential AAV restriction factors**

MAGeCK analysis (207, 208) of the GFP+ cells identified several factors enriched in the cells with high mean fluorescence intensity [Fig. 4.4.A]. ACSL6, Acyl-CoA Synthetase Long Chain Family Member 6, catalyzes the formation of Acyl-CoA from fatty acids and may be playing a role in lipid metabolism, influencing AAV entry at the endosomal membrane. LETM1, LETM1 Domain Containing 1, has been suggested to have a role in p53 regulation and tumorigenesis. CALN1, Calneuron 1, negatively regulates golgi-to-plasma membrane transport, deletion of which could potentially alter the trafficking pathways upon AAV transport to the nucleus. SSH3, Slingshot Protein Phosphatase 3, plays a role in actin dynamics by activating ADF/cofilin proteins, which may also influence and alter the AAV entry trafficking pathways. The most significant hit in the GFP+ subset is TMEM125, an uncharacterized transmembrane protein. Individual
sgRNA(s) targeting several of the top hits from the GFP+ selection were introduced to Huh7 AAVR KO Cas9 cells using a lentiviral vector, then puromycin selected cells were assessed for rh32.33 transduction level using a luciferase assay. Data presented are fold increase in RLU relative to parental cell line (Huh7 AAVR KO Cas9). Several sgRNA transduced cell lines demonstrated increased transduction relative to the parental cell line, most notably TMEM125 and GMEB2 (glucocorticoid modulatory element binding protein 2), which each showed roughly a 100-fold increase in

Figure 4.4: Identification of potential AAV restriction factors. Robust Rank Aggregation [RRA] analysis identifies genes enriched in cells highly expressing GFP, suggestive of potential AAV restriction factors (A). Fold increase in transduction of 10,000 VG/cell rh32.33.CMV.Luciferase.SVPA in CRISPR edited polyclonal cell population. Polyclonal cell lines were generated by transduction of Huh7 AAVR KO Cas9 cells with lentivirus encoding sgRNAs targeting individual genes identified in rh32.33 GFP+ cell population.
transduction [Fig. 4.4.B]. While it is currently unclear whether any of these individual proteins play a role in entry or restriction of AAV, these hits and their role in AAV biology warrant further investigation.

**Identification of potential AAV entry factors**

Analysis of the GFP- cell population identified several genes that were enriched in the GFP- population, one of the most significant of which was GPR108 [Fig. 4.5.A]. This

![Figure 4.5: Identification of AAV entry factors by CRISPR screen.](image)

**Figure 4.5:** Identification of AAV entry factors by CRISPR screen. RRA of first (B) and second (C) round of GFP- transduction. X axis: individual genes within GeCKO library, grouped by functionality. Y axis: significance of hit based on RRA analysis. Bubble diameter: number of individual sgRNAs per gene enriched in the selected population, relative to unselected control.
gene was even further enriched in the analysis of the second round of transduction [Fig. 4.5.B], as well as other genes that were highly enriched such as NEU1 and CTSA. Importantly, the significance of the top hits increased to a p-value of near $10^6$, while other genes stayed the same, around a significance value of $10^3$. This suggests that the second round of transduction was extremely important for the enrichment of rh32.33 entry factors.

**NEU1 and CTSA are required for entry of alternate entry pathway serotypes**

**rh32.33 and AAV4**

Since NEU1 and CTSA exist in a complex together and NEU1 stability and conformation is dependent on CTSA (204, 205), we wanted to test whether both of these proteins were important for the alternate AAV entry route. We tested two AAVR independent serotypes, rh32.33 and AAV4 in previously published Mouse Embryonic Fibroblast (MEF) cell lines derived from either NEU1 WT or KO mice, or CTSA WT, Heterozygous (HET), or KO mice. Both rh32.33 and AAV4 showed a loss of transduction in the NEU1 and CTSA KO cells, with little or no effect being observed in the CTSA heterozygous cells [Fig. 4.6.A,B]. We tested several other AAV serotypes, including AAV5, which uses sialic acid as an attachment factor. Although NEU1 is involved in sialic acid glycan biology, we did not observe any difference in transduction of any AAVR dependent serotypes [Fig. 4.6.C-F]. This demonstrates that NEU1 and CTSA are specifically required for AAVR independent entry, and that rh32.33 and AAV4 appear to use the same alternate entry pathway. We additionally aimed to test the effect of NEU1 loss in human cells by introducing either an NEU1 or CTSA specific sgRNA
into a variety of Cas9 cell lines. Although we were unable to isolate a monoclonal cell line with complete NEU1 knock-out (data not shown), when cells were tested after puromycin selection in a polyclonal context we were able to see a large decrease in

Figure 4.6: NEU1 and CTSA are uniquely required for entry of AAVR independent serotypes. Mouse embryonic fibroblast (MEF) cells from mice that are WT, NEU1 knock-out [KO], CTSA heterozygous (HET) or CTSA knock-out [KO] transduced with AAVR independent serotypes rh32.33 or AAV4 (A,B) or AAVR independent serotypes AAV5, Anc80, AAV9, or AAV9.PHP-B (C-F) in the presence of hAd5 helper virus. (G) Various human cell lines expressing Cas9 were transduced with a lentivirus containing CTSA or NEU1 specific sgRNA and puromycin resistance gene, selected with puromycin, followed by transduction of different AAV capsids expressing CMV.Luciferase.SVPA transgene.
rh32.33 transduction in multiple NEU1 sgRNA transduced cell lines but no decrease for any other serotypes tested [Fig. 4.6.G]. This suggests that NEU1 is required for AAVR independent entry in both human and mouse cells.

**Enzymatic activity of NEU1 is required for alternate pathway entry**

Because NEU1 is an enzyme and CTSA, also identified in our entry screen [Fig. 4.5.C], is required for maintaining the catalytically active conformation of NEU1 (203, 209), we wanted to test whether enzymatic activity of NEU1 is required for its function in rh32.33 and AAV4 entry. We used two different sialic acid analog neuraminidase inhibitor compounds, Zanamivir (210) and DANA (211), to do a dose response on Huh7 cells and assess the effect on entry of different AAVR dependent and AAVR independent serotypes. We tested two different AAVR dependent serotypes, AAV5 and Anc80, and two AAVR independent serotypes, rh32.33 and AAV4. Importantly, AAV4 and AAV5 were examined because both AAVs use sialic acid as an attachment factor (73, 212) yet differ in their AAVR dependence. A short, 1h pre-treatment of cells did not show any decrease in transduction (data not shown). However, pre-treatment of Huh7 cells for 24 hours with either inhibitor drastically decreased rh32.33 transduction by roughly ten-fold, as well as slightly decreased AAV4 entry [Fig. 4.7.A,B]. The requirement for long pre-incubation with neuraminidase inhibitors to show a decrease in AAVR independent entry suggests a that the entry defect may be secondary to NEU1 and CTSA function, in the sense that NEU1 activity may be regulating activity of another protein or cellular process required for entry. Neither of the AAVR dependent serotypes,
AAV5 or Anc80, showed a decrease in transduction, demonstrating that the activity of NEU1 is specifically required for entry of AAVR independent serotypes.

Figure 4.7: **Enzymatic activity of NEU1 is required for entry of AAVR independent serotypes.** Huh7 cells subjected to 24 hour pre-treatment at indicated concentrations of neuraminidase inhibitors Zanamivir (A) or DANA (B) followed by transduction of 10,000 VG/cell of the indicated capsid serotype encapsidating a CMV.Luciferase.SVPA transgene. Black: AAVR dependent serotypes. Green: AAVR independent serotypes. Solid line: unknown glycan attachment factor. Dotted line: sialic acid used for attachment.

Because NEU1 and CTSA play a role in cellular glycosylation states we wanted to be sure the observed entry defect was not due to an overall alteration in glycosylation at the cell surface leading to an attachment defect. To do this, we first pre-treated cells for 24h with Zanamivir or DANA, followed by treatment with a recombinant neuraminidase from vibrio cholera to remove any sialic acid that may have accumulated at the cell surface due to NEU1 inhibition. We then added the indicated pre-chilled vector to cells on ice, incubated for 1 hour for the vectors to undergo attachment, washed away unbound vector using ice-cold PBS, then allowed transduction to proceed and assessed vector transduction in the different treatment conditions by fold-change relative to
untreated control cells via luciferase assay. We examined the same 4 vectors used in Figure 4.7, to tease apart the function of NEU1 on attachment verses entry of these different serotypes. After neuraminidase treatment of Zanamivir or DANA treated cells, we were unable to observe a reversal of entry inhibition [Fig. 4.8.A], suggesting that the

Figure 4.8: **NEU1 entry defect for AAVR independent serotypes is not due to a cellular attachment defect.** Huh7 cells were pre-treated for 24 h with 2 mM of the indicated compound, followed by a 2 h treatment with Neuraminidase from vibrio cholera before transduction with rh32.33 (A), AAV4 (B), AAV5 (C), or Anc80 (D)
Figure 4.8 (Continued): encapsidating a CMV.Luciferase.SVPA transgene. (E) qPCR of cell-bound viral genomes on WT and mutant MEF cell lines for the indicated serotypes.

rh32.33 entry defect is not due to altered glycan structure at the cell surface. In contrast, AAV4 and AAV5 both showed a drastic drop in transduction after treatment with exogenous neuraminidase, as expected due to a loss of their preferred glycan attachment factor, a terminal sialic acid moiety [Fig. 4.8.B,C]. Because Anc80 has no known attachment factor and uses AAVR, we observe no effect of NEU1 inhibition or neuraminidase treatment on overall transduction of Anc80, as expected [Fig. 4.8.D]. These data suggest that the NEU1 and CTSA entry defect is likely not due to global perturbation of glycan structure on the cell surface. To directly assay vector attachment, we used a qPCR based cellular binding assay previously described in Chapter 2 to measure AAV attachment to NEU1 and CTSA WT or KO MEF cells. While we were able to demonstrate differences in attachment of different vectors, roughly 100-fold more iVG/cell AAV4 compared to rh32.33 [Fig. 4.8.E], we did not observe a major difference in WT vs. NEU1 or CTSA KO cells for any of the vectors tested. This binding assay demonstrates that a loss of transduction in NEU1 and CTSA KO cells is not due to a defect in attachment at the cell surface and that these proteins likely play a post-attachment role as an entry receptor or part of a multi-protein entry-receptor complex.

**GPR108 is required for entry of AAVR dependent and AAVR independent serotypes in multiple cell types**

The most significantly enriched gene identified in this screen was an uncharacterized 7transmembrane G-protein coupled receptor-like protein, GPR108 [Fig. 4.5.A,B].
Interestingly, this protein was also identified as a potential entry factor in the initial haploid screen that identified AAVR (93). This suggested to us that GPR108 may be important not only for rh32.33 entry, but for entry of other AAV serotypes as well. We therefore generated a GPR108 KO Huh7 cell line and tested a panel of extant serotypes.
Figure 4.9 (Continued):
CMV.eGFP.T2A.Luciferase.SVPA (AAV Anc83, AAV Anc110, AAV2) transgene. (C)
Transduction level of indicated serotypes in AAVR KO, GPR108 KO, or double KO cells
relative to WT Huh7 cells, with or without helper virus. 10,000 VG/cell
CMV.Luciferase.SVPA transgene.

as well as putative ancestral intermediate capsids [Fig. 4.9.A] for GPR108 usage via
luciferase assay. Transduction of all tested serotypes except AAV5 was greater than 10
to 100-fold decreased in the GRP108 KO cells compared to WT Huh7 cells [Fig. 4.9.B].
In cells deleted for both AAVR and GPR108 there is a complete loss of transduction of
all serotypes, whether cells were pre-infected with a helper virus or not [Fig. 4.9.C].
Loss of transduction upon GPR108 KO is also observed in H1 HeLa cells [Fig. 4.10.A],
suggesting that requirement of this cellular entry factor is conserved in all AAV
transducible cell lines.

**AAV entry can be rescued by stable or transient transfection of GPR108**

To confirm the GPR108 KO defect is due to a loss of GPR108 protein expression, we
stably re-introduced the GRP108 cDNA using a lentiviral vector into H1 HeLa GPR108
KO cells. Stable re-introduction was able to rescue transduction of all tested GPR108
dependent vectors, but KO and rescue had no effect on the overall transduction level of
the GPR108 independent AAV5 [Fig. 4.10.A]. Because there are no functional
antibodies available for detection of GPR108, we designed a construct containing a 3x
alanine-glycine linker at the c-terminus, followed by a flag-tag for detection of GPR108
protein expression. This construct as well as a flag tagged homolog, GPR107, were
subcloned into pcDNA3.1(-) and transiently transfected into WT or GPR108 KO Huh7
cells, followed by transduction with a variety of GPR108 dependent and independent
serotypes. Although there was not a full rescue of transduction to wild type levels, there is a clear rescue phenotype observed from transfection of GPR108, but not GPR107.

Figure 4.10: **GPR108 entry defect can be rescued in multiple cell types via stable or transient transfection.** (A) H1 HeLa cells deleted for GPR108, then stably transduced with GPR108 lentivirus, followed by transduction of the indicated serotypes at 10,000 VG/cell with and without helper virus. CMV.Luciferase.SVPA transgene. (B,C) Huh7 WT or GPR108 KO cells transfected with flag-tagged human or mouse GPR107 or GPR108 followed by transduction of the indicated serotype in the presence of hAd5 helper virus. 10,000 VG/cell CMV.Luciferase.SVPA transgene.
[Fig. 4.10.B,C] for all GPR108 dependent serotypes. These data demonstrate that GPR108 protein expression is required for the entry pathway of most AAVs aside from the most evolutionarily divergent serotype, AAV5.

**GPR108 usage is conserved in mouse**

As human and mouse GPR107 and GPR108 are highly similar sequences, we wanted

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**Figure 4.11:** GPR108 usage is conserved in mouse cell lines, but is not sufficient for complete rescue of AAV transduction. Transduction of rh32.33, AAV4, AAV5, (A) and Anc80, AAV9, and AAV9.PHP-B (B) in Hepa WT or GPR108 KO cells transfected with flag-tagged human or mouse GPR107 or GPR108. 10,000 VG/cell CMV.Luciferase.SVPA transgene.
to determine whether GPR108 was similarly used to for AAV entry in mouse cells. We used hepa cells, a mouse hepatoma cell line, as an analogous mouse in vitro system to the human Huh7 cells. Interestingly, AAV5 is able to transduce hepa cells to a high level, while other serotypes such as rh32.33 and AAV4 are not [Fig. 4.11.A]. This suggests that the alternate factor AAV5 uses in place of GPR108 is likely highly conserved in mouse, yet mouse GPR108 may not be as highly functional as human GPR108 for some serotypes. We additionally used an sgRNA against mouse GPR108 to generate a hepa GPR108 KO cell line. Of the GPR108 dependent serotypes tested that transduce hepa cells, all demonstrated a 10 to 100-fold decrease in transduction in the hepa GPR108 KO cells compared to wild type [Fig. 4.11.B]. We re-introduced flag-tagged human or mouse cDNAs of GPR108 or a homologous protein GPR107 (213) into hepa GPR108 KO cells, and were able to observe a slight increase in AAV transduction [Fig. 4.11.B]. Interestingly, in human cells, mouse GPR108 is able to rescue transduction to similar levels as the human GPR108 construct [Fig. 4.10.B,C]. It is possible that these constructs were not successful to rescue transduction due to low protein expression levels. We therefore assessed expression of each of these constructs using an anti-flag western blot from transfected cell lysates in human and mouse cells. GPR107 and GPR108 are both relatively uncharacterized proteins predicted to have 7 transmembrane domains, with a large luminal N terminus and short cytoplasmic C terminus [Fig. 4.12.A,B]. GPR107 has been shown to have both a disulfide bond and a furin cleavage site in the luminal N-terminal domain required for its function (214) [Fig. 4.12.A]. Furin cleavage of GPR108 produces two peptide fragments
of roughly 28 and 34 kDa, the larger of which is visualized by anti-flag western blot after transient transfection in Huh7 cells [Fig. 4.12.C].


**GPR108 is not facilitating AAV attachment**

The current understanding of factors involved in AAV transduction primarily exists surrounding factors involved in AAV attachment, and there is little known about the
presence and mechanism of intracellular AAV entry receptors. We therefore wanted to test whether GPR108 is playing a role in AAV attachment or further downstream in the entry pathway. We tested two different capsid surface mutants that have altered tropism or binding properties, alongside their parental AAV capsid in Huh7 and H1 HeLa GPR108 KO cells. First, a luciferase assay demonstrates that a peptide insertion variant of AAV9, AAV9.PHP-B (215), was similarly dependent on GPR108 to the parental capsid, AAV9 [Fig. 4.13.A]. Additionally we tested an AAV2 variant containing point

Figure 4.13: **GPR108 entry defect is not due to an attachment deficit.** (A) Transduction of WT or GPR108 KO Huh7 or H1 HeLa cells transduced with parental capsid AAV9 or surface exposed peptide-insertion capsid AAV9.PHP-B. (B) Transduction of glycan-binding defective AAV2HSPG- and parental capsid AAV2 in WT or GPR108 KO Huh7 or H1 HeLa cells. (C) qPCR binding assay for cell-bound viral genomes in Huh7 WT, AAVR KO, GPR108 KO, or double KO cells, assessed for the indicated capsid serotype.
mutations that ablate binding to the primary AAV2 attachment factor, heparin sulfate (216). While the HSPG- variant had overall decreased transduction in all cell lines tested, transduction of the GPR108 KO cells were 10 to 100-fold decreased compared to their wild-type counterpart, suggesting that GPR108 usage is independent of AAV attachment. While these data suggest that GPR108 does not facilitate attachment, we wanted to test this directly. We therefore employed the same binding assay as described in chapter 2 [Fig. 2.3.A], to assess attachment of GPR108 dependent and independent serotypes. We tested Huh7 WT and GPR108 KO cells, as well as AAVRKO cells and the double KO cells, since AAVR was previously suggested to play a role at the plasma membrane (93). We observed no difference in the number of bound viral genomes per cell in any of the knock-out cell lines for any vector tested [Fig. 4.13.C], yet we were able to detect differences in the number of bound viral genomes for different serotypes, demonstrating that GPR108 is not facilitating attachment.

**GPR108 independence is transferrable and is dependent on the phospholipase-containing VP1 domain of capsid**

In order to further understand the function of GPR108 for entry and how it engages the capsid, we used chimeric capsids to identify the capsid domain that dictates GPR108 usage. Because AAV5 and AAV2 differ in their GPR108 usage, we used generated chimeras between these two serotypes. A previous study used directed evolution in airway epithelial cells to identify a chimeric capsid composed of AAV2 VP1 and AAV5 VP2/3, containing a point mutation that is a functional chimera (177). The authors describe a single point mutation, A581T, observed within the VP3 portion of capsid that
played a major role in this functionality. We therefore designed a set of reciprocal chimeras with and without the analogous point mutation to determine which region of capsid dictates GPR108 usage [Fig. 4.14.A]. We then tested these chimeras in Huh7.

Figure 4.14: GPR108 dependence is dictated by the VP1 unique region of capsid and is transferrable to other AAV serotypes. (A) Design of chimeric capsids used to determine GPR108 usage domain (B) Transduction of Huh7 WT, AAVR KO, GPR108 KO, or double KO cells with the indicated WT or chimeric capsids. 100 μL crude vector prep, plus hAd5 helper virus, CMV.eGPF.T2A.Luciferase transgene.

WT, AAVR KO, GPR108 KO, or double KO cells. As AAV2 and AAV5 both require AAVR, we observe the expected loss of transduction for all tested serotypes in the AAVR KO and double KO cell lines [Fig. 4.14.B]. Interestingly, both of the chimeras containing the VP1 unique region of AAV5 were able to transduce GPR108 KO Huh7.
cells to a similar level as WT cells. Residue 581 did not appear to play a major role in GPR108 usage, although it did have a small effect on overall transduction levels. These demonstrate that the VP1 unique region of AAV dictates GPR108 usage, and that this cellular functionality is transferrable to other AAV serotypes.

**IV: Discussion:**

This work is one of the first instances of a highly stringent genome-wide screen to identify viral entry factors being used to understand the entry pathway of a gene therapy vector. We were able to successfully identify and characterize three novel host cell entry factors, used by both AAVR independent and some AAVR dependent AAV serotypes. Our entry screen also highlights several potential host cell restriction factors which are of interest for further investigation as potential targets to increase transduction of AAV gene therapy vectors. The highly conserved usage of two entry factors, AAVR and GPR108, demonstrate that most AAVs appear to share the same entry pathway. Investigation of the sequence space surrounding the divergent serotypes which use alternate factors will likely lead to novel capsids with unique cell and tissue targeting properties, allowing targeting of novel tissue or cell types not previously accessible to the extant AAV serotypes. We additionally present a novel multi-factor entry mechanism in which most AAVs bind AAVR and require it for proper trafficking, followed by requirement of GPR108 for endosomal escape, yet rh32.33 use an alternate receptor complex comprised minimally of NEU1 and CTSA in place of AAVR, and AAV5 requires an as-yet unidentified factor in place of GPR108 for endosomal escape [Fig. 4.15].
Figure 4.15: Model and summary of current understanding of usage of AAV cellular entry receptors, by serotype. Most AAV serotypes [red] require both AAVR and GPR108 for cellular entry in human and mouse. AAV5 [blue] uniquely uses an alternate domain of AAVR, as well as a currently un-known co-receptor for endosomal escape. AAV4 and rh32.33 [green] use a minimal receptor complex of NEU1 and CTSA, as well as the GPR108 co-receptor for endosomal escape.

Many viruses encounter cellular restriction factors that impede their entry and gene expression, yet the existence and identity of AAV restriction factors remains unclear.

Several siRNA screens have attempted to identify AAV restriction factors as potential targets to modulate the efficacy of gene therapy vectors (178-180), yet these screens have met with little follow-up characterization and have little overlap between the
different experimental screens. In this screen, Cas9 cells transduced with TMEM125 or GMEB2 sgRNA showed a greater than 100-fold increase in transduction in a Luciferase-based transduction assay. Because these are not single-cell clones, it is currently unknown what proportion of the population of these cells contain a complete deletion in the gene of interest, but an increase in transduction of such large magnitude in a polyclonal cell population suggests that these genes play a role in preventing AAV entry or gene expression. TMEM125 is an uncharacterized transmembrane with no known homology. GMEB2, however, is also known as PIF (Parvovirus Initiation Factor), and has been implicated in genome replication for other paroviruses outside of the dependovirus subfamily, such as Minute Virus of Mice (217). PIF/GMEB2 has been described as a required replication initiation factor for MVM that binds the minimal replication origin (218) and is required for rolling circle replication (219). While there are many differences in the genome replication and gene expression between dependoviruses and autonomous paroviruses, it is curious that this protein appears to play a role in both subtypes of parovirus. Characterization of the role of GMEB2 in AAV genome replication and genome expression both in a wild-type and vector context may help inform strategies for modulating the genome to favor expression of the vector genome. Investigation of both TMEM125 and GMEB2 will likely open avenues to modulate and potentially increase AAV vector efficacy, leading to decreased production costs and increased safety profiles due to decreased vector dose.

We were able to demonstrate a requirement of NEU1 and CTSA specifically for AAVR independent serotypes, which is not due to a defect in cellular attachment. There is still
ongoing work to investigate the exact mechanism by which NEU1/CTSA facilitate entry and whether there are any other factors involved in this entry complex. As a multi-functional protein that is part of a variety of protein complexes and can alter activity of other transmembrane proteins such as the insulin receptor (220), some G-protein coupled-receptors (221), and toll-like receptors (222, 223). It is therefore of interest to determine whether these proteins may be in complex with or alter the activity of another protein identified in this screen, such as FZD4 of SYNGR2 [Fig. 4.5.B], that may be functioning together as an entry receptor complex. Because a long pre-treatment of cells with neuraminidase inhibitor is required to observe an entry defect [Fig. 4.7.A,B], this is suggestive of NEU1 regulating activity of another protein. This is supported by similar cellular attachment levels observed in cells lacking NEU1 or CTSA, demonstrating that NEU1 inhibition is not causing a global glycan alteration and attachment defect [Fig. 4.8.E]. Additionally, it is known that NEU1 regulates calcium-dependent lysosomal exocytosis by altering glycan composition and activity of LAMP1 (224), which may cause an inability of other receptors/co-receptors to localize to the cell surface. Preliminary co-immunoprecipitation data suggest that AAV4 and rh32.33 may bind to NEU1, either directly or indirectly in cell lysates, but the NEU1 antibody may cross-react with other human neuraminidases, and such we are unsure whether this interaction is unique to NEU1 (data not shown). This data is not conclusive but suggests a physical interaction of AAV capsid with NEU1 or an NEU1-containing complex.

It is currently unclear whether CTSA is playing an enzymatic or protective role as an AAV entry factor. Interestingly, NEU1 itself is glycosylated at 3 different sites that are
required for its activity, and CTSA can rescue an NEU1 glycosylation defect (225). To determine whether enzymatic activity of CTSA or solely the NEU1 protective function of CTSA is required for function of the NEU1/CTSA complex for AAV entry, it is of interest to test the effect of a previously published CTSA mutant that is defective for enzymatic activity yet functional for formation of the lysosomal multienzyme complex (226).

Additionally, CTSA cleaves the autophagy factor Lamp2a acting as a negative regulator of autophagy such that CTSA deficient cells undergo autophagy (227). Because there are major rearrangements in the lysosomal compartment during autophagy, it is of interest to determine the effect this may have on the endosomal pathway of rh32.33 and AAV4, and whether autophagy or autophagy-related genes play any role in the entry pathway of these AAVs.

These studies have large implications for the translation of AAV-based gene therapies, especially in the lysosomal storage disorder field. Point mutations in NEU1 and CTSA cause the lysosomal storage disorders sialdosis (228, 229) and galactosialidosis (230), respectively. There are currently ongoing efforts to develop AAV-based gene therapies for these (231, 232) and other lysosomal storage disorders. This study is the first demonstration of how AAV capsid choice may drastically influence gene therapy outcome in a disease context, as rh32.33 or AAV4-based gene therapies would likely be unable to transduce cells and tissues of patients due to this entry defect. We are currently testing transduction of the NEU1/CTSA dependent serotype rh32.33 in an NEU1 KO mouse model (233), and aim to also investigate the efficacy of these and other vectors in transgenic knock-in mouse models of sialidosis (232). This is the first
demonstration of a cellular entry factor that may influence success and translation of an AAV-based gene therapy based on a cellular entry mechanism.

As an uncharacterized protein, there are still huge holes in our knowledge of GPR108 function in cell biology and as an AAV entry factor, yet our studies are starting to shed some light on its AAV entry function. The lack of major differences in cellular attachment of AAV to GPR108 KO cells [Fig. 4.13.C] suggest that the role of GPR108 is internal, and as such is a bona-fide entry factor. This is further supported by the observation that the major GPR108 usage determinant is the VP1 unique region [Fig. 4.14.B], a domain that is shielded within the capsid until it is in the acidic pH of the endosome. Because the major known functional activity of VP1 is the phospholipase activity (57), we can suggest with some certainty that the function of GPR108 is to facilitate endosomal escape. Interestingly, although there is no structure of a homologous protein available, the Phyre2 protein structure prediction server (234) determined that the most highly-related structure is human rhodopsin, suggesting that the 7-TM domain of GPR108 may actually make a pore within the membrane that could facilitate endosomal escape of the capsid or the AAV genome. This hypothesis should, however, be directly measured. To this end, we are carrying out cellular fractionation and viral genome qPCR assays in GPR108 KO cells to determine where the defect lies within this entry pathway. Importantly, genome-containing capsids can assemble from VP3 only, yet are completely defective for entry in the absence of VP1/2 (161), demonstrating that in some manner GPR108 interaction with this domain is absolutely necessary for productive infection. It is additionally of interest to identify the cellular factor AAV5 uses
in place of GPR108. Because VP1 requirement for entry and nuclear import functions is conserved within other parvoviruses (235, 236), the highly divergent AAV5 may use similar cellular factors to other parvoviruses for these entry functions.

In comparison to the human homolog GPR107, GPR108 appears to have some structural and biochemical differences in the functional protein. It is unclear whether the N-terminal luminal domain of GPR108 contains disulfide bonds as GPR107 does (214). Based on observed molecular weight of the tagged cDNA, GPR108 does not appear to have any cleavage site in the N-terminal domain analogous to the GPR107 furin cleavage site [Fig. 4.12.A-C]. Interestingly, tagged human GPR108 is observed at the expected molecular weight of roughly 60kDa, yet the apparent molecular weight of the mouse GPR108 isoform is much higher than the expected molecular weight of 64kDa, and is observed at roughly 75 kDa [Fig. 4.12.C]. This suggests that although the mouse GPR108 is functional in human cells, it may be post-translationally modified. The regions of GPR108 that contain the most sequence diversity between human and mouse protein sequence are the N and C terminal domains, as well as the first transmembrane domain (Appendix A). It is therefore likely that the major functional domain of GPR108 is within the transmembrane portion. We have designed and are currently testing GPR107/GPR108 chimeric proteins to determine the domains of GPR108 required for its cellular entry function.

It is clear from previous mechanistic studies on AAVR that AAV must traffic to the trans-golgi network for productive infection (93), but it is unclear how and where AAV interacts
with GPR108. We aim to use our recently generated tagged GPR108 constructs for co-immunoprecipitation studies to determine whether GPR108 interacts with either AAVR or AAV capsid. As both AAVR and GPR108 are transmembrane proteins and the transmembrane domain of both AAVR and GPR108 are highly conserved, these proteins may interact within their transmembrane domain. If there is a physical interaction between GPR108 and capsid, it may be difficult to determine since the VP1 domain that dictates GPR108 usage [Fig. 4.14.B] is internalized in the native capsid at neutral pH. Any GPR108 interaction may therefore only be observed upon VP1 extrusion in the endosome (94, 160). Because trafficking to the trans-golgi network is required for AAV entry, we expect AAV may first encounter GPR108 in this subcellular compartment. Since flag-tagged GPR108 is functional to rescue AAV entry in GPR108 KO cells [Fig. 4.10.B,C], we expect this protein to correctly localize within the cell and will determine its localization using immunofluorescence. To further determine the importance of localization in the cell, alternate trafficking domains may be swapped with the natural cytoplasmic c-terminal domain to re-direct the protein to other cellular compartments and determine the effect on AAV entry, as done in previous AAVR studies (93).

Rational modulation of capsid interaction with host cell entry factors may influence vector characteristics to generate capsids with novel functionalities. There is no known AAV sequence, for example, that is independent of AAVR and GPR108, yet there are vectors that are each independent of a single one of these factors. Because VP1 can be swapped between a variety of different serotypes (177, 237), one could envision a
vector in which the GPR108 independence domain is engrafted onto an AAVR independent VP2/3, thus generating a vector that is completely independent of the two canonical entry factors. It has previously been published that other VP1/2 can be swapped between multiple other serotypes, so AAV5 VP1 will likely be able to make functional GPR108 independent chimeras with many other AAV serotypes. Evolutionary analysis of AAV capsid recombination events demonstrated high levels of recombination at the VP1/2 junction (238), suggesting that generation of capsid chimeras at this junction is well tolerated.

AAV targeting differences observed from the known serotypes cannot be explained by our current understanding of AAV entry pathways. Much of the literature is devoted to describing attachment factors, and labeling them entry receptors because of the large deficits in our mechanistic understanding of AAV as a virus. This work is novel in not only its method for studying AAV, but for its identification of multiple major AAV entry factors and description thereof. We believe that further investigation and modulation of capsid interaction with cellular entry factors will lead to unique vector characteristics such as novel tropism that are desired for generation of new and different therapeutics. As only recently identified cellular entry factors, AAVR and GPR108 expression in various cell types and tissues is poorly characterized but expression of these and NEU1/CTSA likely dictate targeting of different AAV vectors. It has been previously reported, for example, that impaired nuclear import and uncoating are thought to be why AAV can not target some cell types (239, 240), potentially because these required entry factors are not expressed in those cells. Interaction of host-cell factors with AAV have
largely been ignored in the development of AAV-based gene therapies, yet our work demonstrates that these factors should not be overlooked when designing vectors or deciding which capsid to use for a particular indication. Our aim is that further understanding of the cellular entry requirements for different AAVs allows improved rational design and implementation of AAV-based gene therapies.

V: Materials and Methods:

Cell culture and transfection

All cell lines were maintained in Dulbecco’s modified Eagle’s minimal medium DMEM (Corning) supplemented with 10% FBS (GE Healthcare) and 100 IU/mL penicillin/streptomycin (Corning) in a humidified incubator with 5%CO₂ at 37°C. All cell lines were a gift from Jan Carrette lab and were previously published (93). Cells were transfected using PolyJet In Vitro DNA Transfection Reagent (SignaGen, Cat#SL100688) using the standard protocol. Expression of flag-tagged constructs was determined by western blotting of whole-cell lysates using mouse anti-flag clone M2 antibody (Sigma F1804) and rabbit anti-beta-actin loading control (Abcam ab8227).

Cloning and plasmid constructs

Flag-tagged GPR107 and GPR108 constructs containing flanking NotI and BamHI restriction sites were synthesized by Genewiz, followed by restriction enzyme subcloning into pcDNA3.1(-) plasmid using NotI and BamHI (NEB) restriction sites. NCBI sequences used for synthesis were as follows: mouse GPR107, BAC26961; mouse GPR108, NP_084360; human GPR107, AAK57695; human GPR108,
Capsid chimeras were generated from AAV2 and AAV5 nucleotide sequence at the VP1 junction demonstrated in (177). Capsid chimeras were synthesized by Genewiz and subcloned into pAAVector2 using HindIII and SpeI restriction sites. Lentiviral plasmids were purchased from Addgene or Sigma. LentiCas9-blast (52962), psPAX2 (12260), pCMV-VSV-G (8454), GeCKO V2A and GeCKO V2B (1000000048 and 1000000049) were purchased from Addgene. Individual sgRNA lentivirus constructs targeting an individual gene used for screen validation and knock-out experiments were purchased from Sigma as QuickPick glycerol stock clones in Sigma LV04 vector backbone.

**Lentivirus production**

Lentivirus was produced from HEK293T cells (ATCC, Manassa, VA), by transient transfection using PolyJet In Vitro DNA Transfection Reagent (SignaGen, Cat#SL100688) using manufacturer’s protocol for lentiviral production. LentiCas9-blast and individual sgRNA-containing lentiviruses were produced in HEK293T cells seeded overnight at 4x10^6 cells per 10cm dish. 1h prior to transfection, medium was changed to fresh pre-warmed D10, followed by transfection of psPAX2, pLentiCas9-Blast or LV04, and pCMV-VSV-G at a 10:10:1 ratio. Medium was changed to fresh D10 6 hours after transfection, and supernatant virus was harvested 48h later, clarified by centrifugation at 2,000 RPM for 5min in Sorvall tabletop centrifuge, and filtered through a 0.45 micron filter. Large-scale GeCKO lentivirus was produced as previously described (206). Briefly, V2A and V2B were produced as individual lentiviral library preps using a large scale tranfection of the protocol described above, in Corning HYPERflask culture
vessels. Supernatant virus was collected at Day 2 and Day 3 post transfection, filtered through a 0.45 micron filter, and concentrated by ultracentrifugation at 24,000 PRM for 2h at 4°C in SW-28 rotor. Concentrated lentiCRISPR library was tittered on Huh7 AAVR KO Cas9 cells by determining % transduced cell survival after 2 days of puromycin selection, relative to untransduced control cells in the absence of puromycin.

**Generation of stable cell lines**

Cell lines were seeded at 1x10⁶ cells per well of a 6 well plate the night prior to transduction. Cells were transduced by spinfection for 30min at 25°C and 2,500 RPM in tabletop using 1mL per well of supernatant lentivirus in the presence of 8 µg/µL Polybrene (ThermoFisher Scientific, Cat#TR1003G). Medium was changed to fresh D10 following spinfection, and one day later stably transduced cells were selected using 5 µg/µL puromycin (Sigma Aldrich, Cat#P9620) for 2 days.

**AAV production and purification**

High titer vectors were produced, purified, and titrated by the MEEI/SERI Gene Transfer Vector Core (http://vector.meei.harvard.edu). Large scale vector preparations were generated by polyethylenimine (Polysciences, Cat#24765-2) triple transfection of pHelp, pAAVector2[Cap], and pCMV.Luciferase.SVPA, pCMV.eGFP.T2A.Luciferase, or pCMV.eGFP.WPRE.bGH transgenes in a 2:1:1 ratio. 520 µg total DNA was transfected in ten-layer hyperflasks using a PEI Max:DNA ratio of 1.375:1 (w/w). 3 days after transfection, vectors were concentrated by tangential flow filtration and purified by iodixanol gradient ultracentrifugation as previously described (148). Chimeric and point
mutant viral vectors were produced on a smaller scale as crude viral preparations by same transfection method in 10 cm cell culture plates. Three days after transfection, cells and supernatant were collected, subjected to three freeze-thaw cycles, then crude virus preparation was clarified by centrifugation for 10 min at 10,000 RPM in a ThermoScientific FIBERLite F15-8x50cy rotor at 4°C.

AAV genome titration

DNase1-resistant viral genomes of iodixanol purified vector preps were quantified by TaqMan qPCR (ThermoFisher, Cat# 4304449) using primer/probe set detecting CMV promoter. Vector purity was assessed by SDS-PAGE electrophoresis.

AAV transduction

All luciferase transduction assays were done by seeding 10,000 cells per well in black-bottom 96 well plates overnight. When indicated, cells were pre-incubated with 200 pfu/cell of WT hAd5 (University of Pennsylvania Vector core) in D10 for two hours, then hAd5 containing medium was removed prior to transduction. Cells were transduced with either AAV at $1 \times 10^4$ VG/cell in 50 μL serum-free DMEM (AAVR rescue experiments) for 1h at 37°C, then D10 was added to a total volume of 200 μL, or 100 μl per well of crude virus prep (chimeric and point mutant capsid experiments) was added for 1h at 37°C, removed, then D10 was added. Transduction levels were analyzed by luciferase assay 48h post-transduction.
Luciferase assays

2 days post-transduction, cell culture medium was removed and cells were lysed in 20 
µL per well of 1x Reporter Lysis Buffer (Promega, Cat#), then frozen at -80°C. After 
thaw, ffLuc expression was measured in Relative Light Units/s on a Synergy H1 Hybrid 
Muli-Mode Microplate reader using 100 µL luciferin buffer [200 mM Tris pH 8, 10 mM 
MgCl2, 300 µM ATP, 1x Firefly Luciferase signal enhancer (Thermo Cat#16180), and 
150µg/mL D-Luciferin].

Entry screen

Huh7 AAVR KO Cas9 cells were transduced with concentrated V2A or V2B lentivirus at 
an MOI of 0.3 in 6-well plates by spinfection as described above for 30 min at 25°C with 
8 µg/µL polybrene, followed by incubation at 37°C and 5% CO2 for 1.5, after which fresh 
D10 media was added. Puromycin was added at a concentration of 5 µg/µL 24h post-
transduction to select sgRNA expressing cells. Cells were cultured with puromycin for 1 
week to carry out selection and allow editing to occur before selection with AAV. 30 
million cells from each half of the mutagenized library (V2A and V2B cells) were 
transduced with 100,000 VG/cell rh32.33CMV.eGFP.WPRE. Cells were transduced in a 
total volume of 10 mL serum-free DMEM in each of two 15 cm plates for 1h followed by 
addition of 10mL DMEM 20% FBS and cells were split the following day. Three days 
post-transduction, cells were collected for FACS sorting by trypsinization, spun in a 
table-top centrifuge at 2,000 RPM for 5 min, then resuspended in PBS (without calcium 
and magnesium) with 5mM EDTA. FACS sorting was done at the Massachusetts 
General Hospital Flow Cytometry Core (Simches Research Building) on a BD FACSaria
Fusion Cell Sorter instrument. Cells were collected into DMEM supplemented with 20% FBS and Pen/Strep. Selected cells were expanded and genomic DNA was extracted from a total of $10^7$ cells per sample. GFP negative cells from each half of the library were split in half and either sequenced or subjected to a second transduction and FACS sort using the same transduction protocol.

**Genome extraction, Illumina barcode addition, and deep sequencing**
Genomic DNA from control (unselected) or selected cells was extracted using a Qiagen Blood & Cell Culture DNA Midi Kit (Cat. No. 13343). Barcode addition and Illumina adapter addition was carried out as previously described (206). Briefly, a two-step PCR was carried out using sample-specific primers to specifically amplify sgRNA sequence and distinguish samples during multiplexed sequencing on an Illumina MiSeq machine as described (206).

**Entry screen analysis**
After sequencing, raw reads were mapped to known sgRNA sequences using the MaGECK analysis pipeline. Significance values were determined for the entire library after normalization to control population within each half of the library (V2A and V2B), and data is reported as raw p-value without multiple test correction.

**Isolation of single-cell knock-out clones**
Cas9 cells were transduced with lentivirus expressing individual targeting sgRNA (LV04 constructs) as described in "generation of stable cell lines." After at least 1 week of
puromycin selection, individual cell clones were plated by limiting dilution in 96-well plates in DMEM 20% FBS plus non-essential amino acids and Pen/Strep to increase cell survival. 2-3 weeks after plating single-cell clones were expanded and screened for knock-out.

**Cell-binding assay**

The indicated cell lines were plated on 24-well plates at $5 \times 10^4$ cells per well overnight. Cells were placed on ice for 10 minutes, then $10^9$ VG per well pre-chilled vector was added in a total volume of 200 µL per well. Vectors were allowed to bind cells on ice on an orbital shaker platform for 1h. Following binding, cells were washed 3x with ice-cold PBS with Mg$^{2+}$ and Ca$^{2+}$ then either 100 µL PBS per well was added and plates were frozen at -80°C. Binding assay plates underwent 3 freeze-thaw cycles, prior to resuspension and viral genome quantification by qPCR as described above using CMV primer/probe.

**NEU1 inhibition experiments**

10,000 cells per well were plated in 96-well plates 1 day prior to inhibitor treatment. Cells were incubated with the indicated concentration of Zanamivir (Sigma SML0492) or DANA (EMD Millipore 252926) for 24h prior to transduction in a total volume of 100 µL D10. When indicated, control or inhibitor treated cells were treated with 50 mU/mL Neuraminidase from Vibrio cholera Type III (Sigma Aldrich, Cat#N7885) in serum-free DMEM, followed by AAV transduction as described.
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Chapter 5:

General Discussion
I: Classification of cellular factors used for AAV entry

Currently most commonly referenced AAV receptors, such as heparin sulfate (174) or HGFR (80) for example, influence the overall magnitude of transduction but are not absolute requirements for AAV entry, and as such cannot be called “receptors.” We propose that instead these cellular factors be re-categorized as “attachment factors.” An analogous example to be considered is the well-characterized receptor usage of HIV-1, which uses heparin sulfate proteoglycan as an attachment factor (241), CD4 as a receptor (242, 243), and subsequent contact with CXCR4 (244) or CCR5 (245) as co-receptors to facilitate fusion/entry of the virus. It is currently unknown if AAV entry is facilitated by a co-receptor after engagement with AAVR, but further studies of the mechanism by which AAV engages GPR108 may shed some light on GPR108’s potential role as a co-receptor.

II: Identification of novel AAV entry factors and outstanding mechanistic questions

Our discovery of an alternate AAV entry pathway used by AAV4 and rh32.33 [Fig. 2.1] led us to investigate alternate entry pathway requirements. A CRISPR screen [Fig. 4.1] identified two novel entry factors, NEU1 and CTSA [Fig. 4.5.B], required specifically by AAVR independent serotypes [Fig. 4.6]. Lack of an attachment defect [Fig. 4.8.E] demonstrated that these factors may be part of a multi-protein receptor complex which functions downstream from attachment. As multi-functional proteins however, the observed entry defect may be due to a secondary defect caused by NEU1/CTSA dysfunction. Due to the many regulatory functions NEU1 has for other cellular proteins
and lysosomal exocytosis (224), it is of interest to determine whether NEU1 may be modifying activity or preventing proper localization of another protein identified in this screen such as FZD1 or SYNGR2 [Fig. 4.5.B]. It is of interest to determine how AAV4 and rh32.33 capsids may bind to this complex to serve the receptor function, since they are unable to bind AAVR [Fig. 2.4.A,E].

As an uncharacterized protein, there are many questions related to GPR108 function that have yet to be determined. Domain architecture is likely similar to GPR107, with a luminal N-terminal domain, a 7 transmembrane domain bundle, and a cytoplasmic C-terminal tail. GPR108 does not appear to be proteolytically cleaved in the N-terminal domain as GPR107 is [Fig. 4.12]. It is unclear whether either of these proteins possess multimerization or signaling functions like those possessed by other g-protein coupled receptors, and if so whether this is required for GPR108 function as an entry factor or if AAV may be able to activate those functions for downstream cellular effects. Multimerization may also occur between other entry factors or entry receptors such as AAVR to facilitate entry. Co-localization, co-immunoprecipitation, and functional studies are ongoing using GPR107/GPR108 chimeras to begin to answer these questions.

III: Capsid domains dictating usage of cellular entry factors and implications thereof

Point mutations [Fig. 3.2] and chimeric viruses [Fig. 3.1] demonstrate that AAVR likely has a large receptor footprint to engage AAVR [Fig. 3.3]. Unpublished structural data recently presented a co-cryo-EM structure of AAV2 in complex with a small PKD1-PKD2
construct. This structure demonstrated a large density roughly the size of one PKD domain mapped at low resolution to the slope just outside of the three-fold spikes, in a similar region to the mutated residues that increased AAVR independent entry of AAV6.2 [Fig. 3.2.D]. Direct binding of capsid to AAVR and detection of AAVR at the cell surface (93, 103) suggests that this is the first protein receptor encountered during the AAV entry pathway. It is unclear exactly where on the capsid the alternate AAV receptor complex (NEU1/CTSA) may bind, but it appears to be within VP3, outside of HVR IV and V [Fig. 3.1]. While mutagenesis and rationally designed chimeras have narrowed down the receptor binding sites, another method such as DNA shuffling (116) may be more useful in determining receptor usage, by identification of overlapping regions required for receptor usage as done previously to identify a functional domain in rh10 (246).

Because the VP1 portion of capsid that is initially internalized determines GPR108 usage [Fig. 4.14], there is likely a structural rearrangement that must occur before engagement of GPR108. It is thought that VP1/2 is extruded through the five-fold pore while in the endosome, and maintained in this conformation during trafficking to the nucleus (94), as is the case for other paroviruses (247-249). This suggests that GPR108 may act as a co-receptor to facilitate endosomal escape or stabilize the VP1/2 extruded capsid intermediate for downstream entry steps. There is likely an active transport mechanism out of the endosome, since the phospholipase domain is extruded from the capsid but likely does not cause complete lysis of the endosome to release the vector into the cytoplasm (250).
IV: Human and animal sequence variation and entry factor function

AAVR polymorphisms causing a loss of function, as observed from Anc80 transductions, [Fig. 3.4.B,C] show proof-of concept that natural human variation may influence gene therapy outcomes due to decreased or defective engagement of required entry receptors/entry factors. The presented loss of function SNPs are low prevalence, but there may be higher prevalence of loss of function SNPs for other entry factor genes such as GPR108, NEU1, and CTSA. The existence of human polymorphisms in NEU1 and CTSA leading to lysosomal storage disorders (228, 230) highlights that these loss of function SNPs do exist within the human population and that considering human polymorphisms may be an important consideration when designing AAV-based gene therapies.

While it is clear that AAVR-dependent entry is conserved in other species, as shown in a knock-out mouse model (93), it is unclear whether relative activity of AAVR or other entry factors will influence how accurate of a predictor for human outcome different animal models are. Mouse and dog AAVR do not appear to be functional in human cells [Fig. 3.5], but this may be due to low expression levels of these constructs. Alternatively, there may be another co-factor required to stabilize these variants that is not compatible with AAVR in the human cell line. Conversely, mouse GPR108 was similarly functional to human GPR108 to rescue transduction in human cells lacking GPR108 [Fig. 4.10.B,C]. GPR108 usage is conserved in mouse, as GPR108 knock-out in hepa cells causes a loss of transduction of all serotypes (aside from the GPR108 independent
AAV5) that can transduce mouse cells [Fig. 4.11.B]. It is curious that AAV5 can transduce mouse hepa cells to a much higher level than other tested AAVs [Fig. 4.11.A], and is the only serotype that is GPR108 independent [Fig. 4.9.B]. This suggests that the alternate entry factor used by AAV5 may either be more highly conserved in mouse or more active in mouse. Based on these data, relative targeting levels of different capsid serotypes tested in a mouse model may not be translatable to humans due to the level of conservation of the entry factors required by these different serotypes.

V: Implications for adeno-associated viral vector design

As recently characterized proteins, AAVR and GPR108 expression levels in various tissues and cell types and how this dictates serotype tropism are poorly understood. In vitro data demonstrates that increased AAVR expression can increase transduction of some, but not all, serotypes [Fig. 3.6]. This suggests that the expression level of AAVR, GPR108, NUE1, CTSA, and other potential entry factors in a target cell will dictate which vector is best suited for an indication, based on relative requirement of these different factors.

Many studies have attempted to change AAV tropism by attempting receptor re-targeting strategies (251-256). These studies however often generate small differences in tissue-specific tropism, generating more shades of grey rather than an “on/off switch” for a particular tissue or cell type as would be desired for a highly efficient and targeted gene therapy. Loss of transduction in AAVR KO [Fig. 2.1.B], GPR108 KO [4.9.B], NEU1
KO [Fig. 4.6.A,B], or CTSA KO [Fig. 4.6.B] for individual vectors requiring these proteins is much more of an “on/off” switch that would be desired for tissue-specific targeting, and which could potentially be harnessed for development of therapies. We have demonstrated that engraftment of the VP1 domain of AAV5 onto an otherwise GPR108-dependent serotype (AAV2) renders it completely independent of GPR108 [4.14]. This opens the potential to engineer any other known AAV serotype to target a cell or tissue type low or lacking GPR108, by routing the capsid through another entry mechanism. Upon more extensive mapping of capsid-binding domains, and defining functional engagement of cellular factors, one can envision rationally engineering a vector of interest to target an individual tissue or cell type based on the target’s endogenous expression levels.

**VI: Concluding remarks**

With the recent identification of novel cellular and viral proteins such as AAVR (93) and AAP (59), there has been a renewed interest in the basic biology of adeno-associated virus. Our aim was to further the understanding of AAV entry biology across the broad spectrum of diverse AAV serotypes to deconvolute the poorly understood cellular factors determining serotype-specific properties such as immunogenicity and tissue tropism. To this end, we have employed a genome-wide screening based method (185, 188) not previously used to study AAV biology to identify multiple cellular factors involved in both conserved and alternate AAV entry pathways. Novel entry factors identified by CRISPR screen demonstrate a conserved entry pathway used by most AAVs, with alternate pathway components being used by the divergent serotypes
AAV5, AAV4, and rh32.33 [Fig. 4.15]. The function of these identified proteins was demonstrated to be post-attachment [Fig. 4.8.E, Fig. 4.13.C], suggesting a role as bona-fide entry receptors/entry factors. We have further characterized the role of AAVR in AAV entry, suggesting that its predominant role is post-attachment [Fig.2.3.B, Fig.4.13.C], and demonstrated that variation within the AAVR coding sequence can lead to a loss of function as an AAV receptor [Fig.3.4.B,C]. Our studies have furthered the molecular understanding of AAV entry mechanism, and the host-cell factors involved in the complex molecular interplay leading to AAV vector transduction. We aim to use this better understanding of AAV-host cell interactions to inform rational design of AAV-based gene therapy vectors.
Appendix A:

Protein sequence alignments of AAV entry factors
AVR alignment continued
II: GPR107 GPR108 alignment
Appendix B:

Sub-neutralizing anti-AAV antibodies affect AAV transduction levels of diverse serotypes
I: Abstract:

A major barrier to successful systemic AAV-based gene therapy is the presence of pre-existing antibodies against the AAV capsid. While it has previously been reported that high concentrations of neutralizing anti-capsid antibodies prevent successful gene transfer, the effect of sub-neutralizing concentrations of antibody or cross-reactive capsid-binding but non-neutralizing antibodies is still unclear. We aimed to investigate the well-characterized antibody effects, antibody-dependent enhancement (ADE) and antibody-dependent intracellular neutralization (ADIN), have on the level of AAV transduction. We used siRNA mediated TRIM21 knock-down in HeLa cells to asses the potential for AAVs to undergo TRIM21-dependent ADIN, and determined that low levels of ADIN may occur for AAV2 in vitro. We assessed a panel of human serum samples for cross-reactive neutralization vs. enhancing effects, and using a dose-response immunization method in mice modeled the potential for AAV to undergo ADE after prior exposure. Interestingly, after immunization all mice including those injected with PBS control showed increased enhancement of Anc80 compared to AAV2, suggesting that there is a capsid-specific enhancement that is dependent on serum, but not anti-AAV antibody. While the current mechanisms of how sub-neutralizing and non-neutralizing antibodies as well as other serum proteins effect AAV transduction in vivo are still poorly understood, our data suggests that these antibodies or serum proteins may alter AAV transduction in a serotype-specific manner and may have implications for in vivo gene transfer.
II: Introduction:

As a human virus, anti-AAV antibodies limit AAV-based gene therapies.

Upon systemic administration into human patients AAV often encounters anti-AAV antibodies present in the serum due to prior AAV exposure. It has previously been shown that presence of AAV neutralizing antibodies is a major factor that determines the success of AAV-mediated gene transfer (16), a measure that is now often used as an exclusion criterion for gene therapy trials. Despite this exclusion criterion, the lack of detectable neutralizing antibodies is still not a universal indicator success, and there is large variability among patient populations (3). Neutralization assay optimization has increased sensitivity and detectability of neutralizing antibodies (257) but this assay is not standardized throughout the field, leading to potential variability in detection of anti-AAV antibodies. These large variations in gene transfer success led us to question what effect sub-neutralizing concentrations of antibodies or serotype cross-reactive antibodies and other highly prevalent serum proteins may have on transduction ability of different AAV serotypes. We have investigated the role these antibodies may play in Antibody Dependent Intracellular Neutralization (ADIN), and Antibody Dependent Enhancement (ADE), two known mechanisms by which sub-neutralizing (binding) antibodies influence the efficiency of entry of other viruses.

Immune responses to AAV vectors must be surmounted in order to achieve sustained therapeutic levels of transgene expression.

As a circulating human virus in the population, 40-90% of the human population has pre-existing neutralizing antibodies (NAbs) against the naturally occurring serotypes of
AAV (258). Even serum titers as low as 1:5 have been shown to drastically decrease AAV transduction in mice and non-human primates (259, 260). Neutralization has been attributed to the failure of an early AAV2-based Hemophilia B trial, in which a patient with a neutralizing AAV2 titer as low as 1:17 failed to see any therapeutic benefit despite administration of a large vector dose (16). In a separate hemophilia trial, several patients in a high-dose group undergoing AAV8-based therapy saw a drop in transgene levels, which corresponded to elevation of liver transaminase levels in the blood. This was presumably due to destruction of transduced cells by a cytotoxic T-cell response (3), but thus far animal models have failed to detect CD8+ hepatocyte destruction in vivo (261). Several attempts have been made to circumvent anti-AAV immunity, including plasmapheresis (262, 263) and saline flushing (263) to remove neutralizing antibodies and immunomodulation by transient immunosuppression (3, 260), with moderate increases in transduction. Efforts to develop novel vectors have also shown promise. A hybrid capsid, titled AAV2.5, has been shown to escape neutralization by NAbs against AAV1 and AAV2 (121), and library generation techniques have allowed selection of capsid variants that escape NAb neutralization (117). Transduction with a chimeric capsid titled rh32.33 has also been shown to demonstrate decreased T-cell responses in the clinic (237, 264). By studying novel AAV entry mechanisms mediated through sub-neutralizing antibodies, our work is relevant to both optimization of neutralization assay sensitivity, and providing new indicators of gene-transfer success in vivo.
Non-enveloped viruses are susceptible to TRIM21-mediated Antibody Dependent Intracellular Neutralization (ADIN).

Using siRNA knock-down, TRIM21 has been shown to mediate antibody-dependent intracellular neutralization (ADIN) of non-enveloped DNA viruses such as adenoviruses (265-267) and picornaviruses (268). TRIM21 is an E3 ubiquitin ligase, composed of a RING domain, two B-Box domains, a coiled-coil domain and a c-terminal PRYPSRY domain. The PRYSPRY domain acts as a high affinity intracellular Fc receptor (269) with broad antibody subclass (IgG, IgM, and IgA) (265, 269, 270) and species specificity (268, 271, 272). TRIM21 has been shown to co-localize with ubiquitin and antibody-coated, fluorescently labeled Ad5 particles during the Ad5 entry process (265). These data, in combination with previously published reports that the RING domain is required for ubiquitination (273) suggest that TRIM21 is facilitating ADIN by ubiquitinating the incoming virions, thus targeting them for proteosomal degradation.

Further work has demonstrated that TRIM21 is constitutively repressed by its B-Box domain but IKKβ and TBK1 de-repress this state through phosphorylation of the RING domain (274). We initially became interested in TRIM21 due to previous reports of inconsistencies in results from neutralization assays used for patient exclusion in clinical trials. Surprisingly, the cell line used in the neutralization assay can make a large difference in the neutralizing titer (257). We therefore wanted to ask whether AAV, also a non-enveloped DNA virus, could undergo ADIN mediated by TRIM21 in the presence of neutralizing antibodies, and whether this could explain discrepancies in the neutralization assay, since there are drastically different TRIM21 expression levels in the different cell lines used [preliminary data from the Vandenberghe lab]. Since ADIN
mediated by TRIM21 has also demonstrated a clear antiviral effect on the pathogenic mouse Adenovirus-1 (266), we also wondered whether ADIN of AAV could be occurring in patients and if this would play any role in gene transfer outcome. Due to the role of the proteasome in ADIN, we additionally hypothesized that TRIM21-dependent ADIN could lead to increased capsid cross-presentation in transduced liver cells, causing the CD8+ T-cell destruction observed in clinical trials.

**AAV transduction is enhanced in the presence of sub-neutralizing concentrations of antibodies.**

Although it has been widely observed in the field, there are few reports in the literature about the mechanism and prevalence of antibody-dependent enhancement of AAV. One paper has observed that mouse anti-AAV2 antiserum is capable of enhancing transduction in monocytic cell lines such as THP-1 and U937, and that blocking FcγRI and FcγRII with anti-FcγRI and FcγRII antibodies decreases this enhancement (275). Enhancement has also been observed in mouse bone marrow macrophages in the presence of mouse serum from mice pre-immunized with AAV2 (276). This group determined that enhancement in these cell lines was due to complement protein C3, as recombinant C3 can bind to AAV2 capsids and heat inactivation abrogated this effect. These two reports, although supporting the ability of AAV to undergo enhancement, do little to define the mechanism of enhancement observed in non-immune cells and whether this enhancement occurs in vivo. For other viruses, enhancement has been reported to occur either through the Fc receptor mediating uptake into immune cells for viruses such as Dengue virus (277, 278), or complement-bound antibodies mediating
entry into non-immune cells such as Ebola (279) and Parvovirus B19 (280). This is observed in situations where there are low affinity antibodies, such as during secondary infection with a different serotype than the primary infection (281). We have observed enhancement in vitro of up to 10-fold from serum samples that are neutralizing against other AAV serotypes. By understanding more about the mechanisms by which sub-detectable levels of antibodies affect transduction, we can develop methods to circumvent their activity in ways that are translatable to the clinic. We have used both in vitro and in vivo studies in mice in an attempt to dissect the role anti-AAV antibodies are playing in AAV entry outside of a classical neutralization mechanism.

III: Results:

AAV is more resistant to neutralization upon TRIM21 knock-down.

Since TRIM21 has been demonstrated to cause increased neutralization of non-enveloped DNA viruses via antibody dependent intracellular neutralization, we wanted to determine whether anti-AAV antibodies may facilitate neutralization of AAV through the same mechanism. We used a neutralization assay optimized in our lab for sensitivity. In this assay, HeLa cells were transfected 2 days prior with either a non-target control siRNA, or a previously published TRIM21-targeting siRNA which we determined to give efficient knock-down [Fig. B.1.A], and these cells were either untreated or treated for 24h with IFNα to increase TRIM21 expression before the neutralization assay was performed. We tested both pooled serum IgA [Fig. B.1.B] and pooled serum IgG [Fig. B.1.C], which have both been previously reported to facilitate
ADIN of adenovirus. We observed a small but reproducible shift in both neutralization

Figure B.1: Trim21 knock-down decreases neutralization activity of pooled and individual human serum samples. (A) Western blot demonstrating TRIM21 expression in HeLa cells transfected with non-target control or TRIM21 siRNA, with or without 24 h IFNα pre-treatment. AAV2 neutralization assay using pooled human IgA (B) or IgG (C) or neutralization assay using individual patient serum samples (D-G).
curves, which was larger for IgG [Fig. B.1.C] than for IgA [Fig. B.1.B]. Since more antibody is required to neutralize AAV2 after TRIM21 knock-down, this suggests that TRIM21 is facilitating antibody dependent intracellular neutralization of AAV at steady-state expression levels in these cells.

**Interferon treatment has no effect on AAV entry or TRIM21-dependent ADIN.**

Because many TRIM proteins are interferon stimulated genes and IFNα treatment has previously been shown to increase TRIM21 expression and enhance TRIM21 mediated ADIN, we wanted to determine the effect of IFNα treatment on TRIM21-mediated ADIN of AAV. Interestingly, although TRIM21 expression is increased upon IFNα treatment in cells transfected with both the non-target control and the TRIM21 specific siRNA, we observed no change in the neutralization curves in any of the samples tested relative to the untreated for the same siRNA construct.

**Individual patient sera facilitate different levels of TRIM21-dependent ADIN of AAV.**

Since only a modest shift in neutralization was observed using pooled human serum samples, we wanted to determine whether different people possessed differential levels of ADIN-causing antibodies, such that a large TRIM21 effect in some samples would be drowned out in the context of pooled serum antibodies taken from many people. We tested several human serum samples that we determined to be neutralizing for AAV2, and repeated our knock-down, IFNα treatment, and neutralization assay within a few dilutions of the previously determined neutralization dilution. As expected, we observed
several serum samples such as sample numbers 39 [Fig. B.1.D] and 41 [Fig. B.1.E] that showed no change in the neutralization curve upon IFNα treatment or TRIM21 knock-down, yet we observed some serum samples such as serum numbers 35 [Fig. B.1.F] and 40 [Fig. B.1.G] which showed a shift in the neutralization curve upon TRIM21 knock-down. These data suggest that there are patient-specific differences in anti-AAV antibodies that may allow AAV neutralization through this unconventional intracellular route.

**Human serum samples show increased enhancement for low-prevalence serotypes**

Although prevalence of neutralizing antibodies against different AAV serotypes around the world have been studied in detail, the effect of non-neutralizing (binding) antibodies, or sub-neutralizing concentrations of anti-AAV antibodies have not been investigated. Binding antibodies have previously been shown to enhance other non-enveloped viruses such as Dengue Virus, so we wanted to determine whether AAV can also undergo antibody-dependent enhancement in the presence of sub-neutralizing antibodies. To do this we started by mining data previously generated in the lab in which the prevalence of neutralizing antibodies was determined from three different human cohorts around the world. Within this dataset, we determined the level of enhancement for each serotype tested and each individual serum sample at a 1:4 dilution, such that samples are only analyzed if they are non-neutralizing [Fig. B.2.A]. We noticed a clear trend that some serotypes such as Anc80, a novel capsid developed in our lab through
ancestral sequence reconstruction, and not present in the circulating population, had a

Figure B.2: **Human patient serum enhances divergent AAV serotypes in a capsid-specific manner.** (A) Relative enhancement of individual patient serum samples (grey) and mean fold enhancement for individuals within each cohort (red) and (B) heat map showing cross-neutralization (red) versus enhancement (green) of different AAV serotypes at a 1:16 serum dilution.
larger level of enhancement than other serotypes. Additionally, a chimera of two rhesus isolates, rh32.33 also showed higher enhancement than serotypes that were isolated from humans such as AAV5, AAV2, and AAV8. We therefore hypothesized that antibodies against high prevalence AAV serotypes may be enhancing transduction of serotypes not circulating in the human population (rh32.33 and Anc80) by an antibody-dependent enhancement mechanism. Interestingly, when we look at a subset of these samples via a heat map and determine the level of neutralization (red) vs. enhancement (green) [Fig. B.2.B], we observe the expected trend in which serum samples that neutralize one serotype (AAV5), enhance other serotypes (Anc80). These data suggest that serum antibodies may be able to neutralize one AAV serotype, while enhancing another AAV serotype.

**Serum enhancement effect is independent of cross-reactive AAV antibodies in a mouse model of ADE.**

Due to our observation that some serum samples neutralize one serotype and enhance a different serotype, we wanted to determine whether we could induce the production of cross-reactive enhancing antibodies in a mouse model. We immunized mice intramuscularly with three different doses of AAV2, (10⁵ VG/mouse, 10⁷ VG/mouse, or 10⁹ VG/mouse) as well as a PBS only control, and bled mice immediately prior to injection (D0) and at days 3, 7, 14, and 21 post-injection. Serum from submandibular bleeds were analyzed for the presence of neutralizing or enhancing antibodies against AAV2 and Anc80 using a standard neutralization assay with two-fold serial dilutions of
serum on HEK293 cells. Due to the low amount of serum collected from some samples,

Figure B.3: **Immunized mouse serum samples enhance AAV transduction, without generating ADE antibodies.** AAV2 (A) or Anc80 (B) enhancement after immunization with dose escalation of AAV2 or PBS control, at different time-points post-immunization.

a 1:16 dilution was the highest serum dilution at which we were able to compare all samples. At this serum dilution we observe the induction of neutralizing antibodies against AAV2 only after immunization with the highest dose, starting at D7 post-injection [Fig. B.3.A]. All mice in the high-dose group sero-converted against AAV2 by Day 21, but none of the serum samples were able to neutralize Anc80 at a 1:16 dilution [Fig. B.3.A]. While we did not observe increased Anc80 enhancement in the immunization
groups, we do observe an overall higher level of enhancement in all serum samples for Anc80 (8-24 fold) than we observe for AAV2 (3-12 fold). This suggests that rather than AAV-specific antibodies causing cross-reactive ADE, we are likely observing the effect of some serum proteins on the capsid causing increased entry of some but not other serotypes.

IV: Discussion:
Our results are consistent with previous reports that IgG has a higher affinity to TRIM21, leading to a higher level of antibody dependent intracellular neutralization with pooled serum IgG [Fig. 2.1.B,C] (270). Additionally, it is known that proteosome inhibition with MG-132 increases ADIN, due to the loss of degradation of the capsid in the presence of anti-capsid antibody (265). MG-132 has been shown to increase transduction of multiple AAV serotypes (98, 282), a mechanism which has been shown to be dependent on the proteasome, as inhibition of serine and cysteine proteases is unable to enhance transduction (283). Due to these reports and data suggesting that surface exposed tyrosines may be ubiquitinated and mutagenesis of these residues increases transduction efficiency of multiple AAVs both in vitro and in vivo (284-288), it may be of interest to examine these mutants as well as proteosomal inhibition in the context of ADIN for AAV.

Although the observed effect is only a difference of roughly 2 to 4 fold of the AAV2 neutralizing titer, it is possible that other serotypes whose entry pathway differ from AAV2 may show a larger effect. TRIM21 mediated ADIN requires the capsid-antibody
complex to be released from the endosome in order to come into contact with cytosolic TRIM21, so any serotypes that may undergo endosomal escape at more neutral pH may have an increased chance of encountering TRIM21. Mechanistic studies of different adenovirus serotypes demonstrate that differences in endosomal trafficking that lead to endosomal accumulation result in increased immune activation and decreased vector efficacy (289, 290). Based on the specific inhibitors used in these experiments, the authors conclude that immune activation is caused by interaction with acid dependent pattern recognition receptors such as TLRs or the inflammasome, however they also may have been preventing release of the capsid to the cytoplasm and interaction with TRIM21. These studies provide rationale for investigating TRIM21-dependent degradation of other AAV serotypes, but thus far serotype specific entry pathway differences are poorly understood and have not been done in a systematic manner. Chapters 4 and 5 of this document study in detail unique entry pathways used by AAV4, rh32.33 and AAV5 which are distinct from most other AAVs and thus may be interesting in determining whether TRIM21 dependent ADIN could occur for these serotypes.

It is of note that some patient serum samples reproducibly demonstrate low levels of TRIM21-dependent ADIN while other serum samples show no effect. This suggests that binding AAV capsid alone is not sufficient to facilitate neutralization through a TRIM21-dependent pathway. Both antibody sub-class and glycosylation state of the antibody have been shown to influence the conformation of the Fc domain leading to different Fc receptor binding and effector function [for review see (291)]. Since the TRIM21
PRYSPRY domain binds the Fc region in a binding mode that has contacts with both Ig domains of the Fc region (269), it stands to reason that these sub-class and glycosylation differences may influence TRIM21 binding and ADIN activity of these antibodies. To our knowledge this is the first description of differential TRIM21 activity caused by specific human serum samples, and understanding these differences may be of interest for both AAV biology as well as understanding the role ADIN plays in the human population for other viruses neutralized through this mechanism.

Although IFNα pre-treatment increased TRIM21 expression, it is surprising that there was no difference in ADIN compared to the untreated condition. Potentially there was not a large enough difference in expression to change the levels of ADIN occurring in our system, since the overall observed effect is only a modest difference in neutralization of AAV. Additionally, AAV is a fairly unique virus in its lack of induction of an interferon response, despite having no known interferon antagonizing proteins. TLR9 and more controversially TLR2 (292) activation is thought to occur in the presence of AAV, but the observed effects are often modest (293, 294). The exact mechanisms surrounding innate immune responses and activation to both TLRs and TRIM21 by AAV thus remains elusive.

Although we were unable to induce cross-reactive Anc80 enhancing antibodies by immunization of mice with AAV2, we did observe an interesting phenomenon that all serum samples enhanced Anc80 to a much greater extent than that of AAV2. This suggests that rather than enhancing antibodies, we may be observing serum proteins
that increase transduction of AAV in a serotype-specific manner. This is consistent with previous reports that human serum albumin (295, 296) and other serum proteins such as LDL and transferrin (296) can bind to AAV capsid and increase transduction both in vitro and in vivo. Alternatively, although mice are kept in a clean facility it is possible that other antibodies in the serum are binding capsid and causing this enhancement effect, despite the lack of an intentional exposure. Also, a recent publication demonstrated that AAV binding antibodies increase transduction of AAV while also demonstrating that other antibodies cause neutralization (297). This either suggests that these enhancing antibodies do exist or that this observation may be due to lingering presence of serum proteins in impure antibody preparations. This mechanism is suggested to be via generation of large immune complexes that cause increased uptake of the AAV complex into cells.

Currently most gene therapy programs including a recent highly successful trial for Spinal Muscular Atrophy (18) exclude patients based on the presence of anti-AAV antibodies using an ELISA-based assay, which would exclude all patients with antibodies regardless of whether they will functionally prevent efficient gene transfer by neutralization, potentially excluding patients that could benefit from these therapies. Although it is still somewhat unclear which proteins are mediating this enhancement effect, it is important to continue to investigate the role of non-neutralizing antibodies in order to both obtain accurate clinical trial results, as well as prevent unnecessary exclusion of patients from trials providing life-saving therapies.
V: Materials and methods:

Cell culture and antibodies
HEK293 and HeLa cells were maintained in DMEM with 10% FBS + Pen/Step. siRNA knock-downs were performed as described previously by transfecting 150 pmol siRNA per well of a 6 well plate using Lipofectamine RNAiMax. TRIM21 siRNA sequence UCAUUGUCAAGCGUGCUGC was ordered from Dharmacon. Two days after transfection, cells were re-plated in block-bottom 96-well culture plates, treated with IFNα for 24 hours, then subjected to neutralization assay. Rabbit polyclonal anti-TRIM 21 antibody was ordered from abcam (ab119895).

Human serum samples and IRB approval
All experiments were approved by the Institutional Review Board (IRB) at Massachusetts Eye and Ear Infirmary. A determination of “Research not involving human subjects” was granted by the Institutional Review Board of each sample provider. Serum samples from individuals without identifiers (except for a general geographical location), and overall healthy population was obtained from MGH Bloodbank, (Boston, Dr. Stowell MD kindly provided the samples); Asturias Bloodbank, Spain (samples were kindly donated by Dr. Alvaro Meana); Red Cross Leuven (Belgium) and Bioreclamation IVT (Florida, samples from individual US donors without identifiers were purchased). All samples were tested and found negative for HIV ½ AB and HCV AB, and non-reactive for HBSAG, HIV-1 RNA, HCV RNA and STS. Serum was shipped by Fed Ex with the required documentation. Proper Personal Protective
Equipment and BL2 conditions for handling these samples and all the measurement were used.

**Neutralization assays.**

TRIM21 neutralization assays were carried out in HeLa cells (without adenovirus) and neutralization assays for enhancement experiments were carried out in HEK293 cells infected overnight with hAd5 at an MOI of 20. $10^9$ (HeLa) or $10^7$ (HEK293) viral genomes per well were incubated with the indicated concentration of IgG, IgA, or serum dilution in a total volume of 50µL serum-free DMEM for 1h at 37°C prior to addition to cells. Immune complexes were incubated on cells for 1h at 37°C with 5% CO₂ then 150µL DMEM, 10%FBS was added and luciferase assay was performed 2 days after transduction.

**Luciferase assay**

2 days post-transduction, cell culture medium was removed and cells were lysed in 20 µL per well of 1x Reporter Lysis Buffer (Promega, Cat#), then frozen at -80°C. After thaw, ffLuc expression was measured in Relative Light Units/s on a Synergy H1 Hybrid Muli-Mode Microplate reader using 100 µL luciferin buffer [200 mM Tris pH 8, 10 mM MgCl₂, 300 µM ATP, 1x Firefly Luciferase signal enhancer (Thermo Cat#16180), and 150µg/mL D-Luciferin].
Mouse studies

All experiments were carried out in accordance with Schepens Eye Research Institute Institutional Animal Care and Use Committee approval protocol number S-441-1018 “Novel Vectors for Therapeutic Gene Transfer in Mice.” For intramuscular injections, C57Bl/6 mice were anesthetized using 2-4% Isoflurane. Hair was removed from skin above the hind limb quadriceps muscle using Nair-fair remover and rinsed clean, followed by application of betadine prior to injection. Vector was injected to the quadriceps in a total volume of 25 µL in 3-4 different injection sites along the muscle. Mouse serum was collected via facial vein bleeding using a 4-5mm lancet and up to 100 µL of blood was collected while animals were hand restrained.

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